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EXERCISE STRATEGIES TO IMPROVE AEROBIC CAPACITY, INSULIN SENSITIVITY AND MITOCHONDRIAL BIOGENESIS

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Exercise strategies to improve aerobic capacity, insulin sensitivity and mitochondrial biogenesis

THESIS FOR DOCTORAL DEGREE (Ph.D.)

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ABSTRACT

Regular exercise plays a key role in the maintenance of health and physical capabilities. Extensive research shows that exercise is an efficient method to prevent diabetes. Both resistance and aerobic exercise training are well known countermeasures for insulin resistance. However, depending on factors like purpose, capability and accessibility, different exercise modes need to be evaluated on both applied and molecular levels. In addition, exercise is the means to improve performance. New training strategies have emerged, like training with low glycogen stores or combining strength with endurance training, and guidelines based on empirical data are needed. Although knowledge of exercise physiology has advanced, much more needs to be learned before we can exploit the full potential of exercise with regard to health and performance. Therefore, the overall aim of this thesis is to provide knowledge of how different exercise strategies improve performance and insulin sensitivity. The mitochondria represent a central part of this thesis considering their key role in both health and performance. Study I was an acute crossover investigation of the effect of exercise with low glycogen levels on markers of mitochondrial biogenesis. Study II investigated the effect of concurrent resistance and endurance training on mitochondrial density and endurance performance. Study III investigated the acute effect of exercise on starvation-induced insulin resistance. In Study IV, the effect of resistance exercise training on health and performance in the elderly was investigated. The main findings were:

- Training with low glycogen levels enhanced the response in markers of mitochondrial biogenesis.
- Adding resistance training to endurance training did not improve mitochondrial density or endurance performance in trained individuals.
- Resistance training for only eight weeks is an efficient strategy to improve strength, heart rate (HR) during submaximal cycling and glucose tolerance in elderly. It also improves muscular quality by increasing mitochondrial and hypertrophy signaling proteins.
- Starvation-induced insulin resistance is attenuated by exercise. Mitochondrial respiration and reactive oxygen species (ROS) production is reduced during starvation. Exercise during starvation reduced glycogen stores and resulted in the activation of enzymes involved in glucose metabolism.
- When exercise was performed during starvation there was an increase in markers for mitochondrial lipid oxidation.

In conclusion, training with low glycogen stores seems to be a promising strategy to increase mitochondrial density. In contrast to our previous acute findings, concurrent training had no effect on mitochondrial biogenesis or endurance performance. Exercise can reverse yet another mode of insulin resistance (starvation) which strengthens its role in the treatment for other states of insulin resistance, e.g. Type 2 diabetes (T2D). Resistance exercise training is an efficient and safe strategy for the elderly to improve health and performance.

LIST OF SCIENTIFIC PAPERS

I. Niklas Psilander**, Per Frank**, Mikael Flockhart, and Kent Sahlin. Exercise with low glycogen increases PGC-1a gene expression in human skeletal muscle. Eur J Appl Physiol 113:951–963, 2013.

II. Niklas Psilander, **Per Frank**, Mikael Flockhart, and Kent Sahlin. Adding strength to endurance training does not enhance aerobic capacity in cyclists. SJMSS, accepted.

III. **Per Frank**, Abram Katz, Eva Andersson, and Kent Sahlin. Acute exercise reverses starvation-mediated insulin resistance in humans. Am J Physiol Endocrinol Metab 304: E436–E443, 2013.

IV. **Per Frank**, Eva Andersson, Marjan Pontén, Björn Ekblom, Maria Ekblom, and Kent Sahlin. Resistance training improves aerobic capacity and glucose tolerance in elderly. Submitted.

CONTENTS

LIST OF ABBREVIATIONS

ACC	Acetyl-CoA carboxylase
ADP	Adenosine diphosphate
AMP	Adenosine monophosphate
AMPK	AMP-activated protein kinase
AS160	Akt substrate of 160 kDa
ATP	Adenosine triphophate
AUC	Area under the curve
BOH	β-hydroxybutyrate
CS	Citrate synthase
CSA	Cross-sectinoal area
ETC	Electron transport chain
FA	Fatty acid
GLUT4	Glucose transporter 4
GS	Glycogen synthase
GSSG/GSH	Glutathione in oxidized (GSSG) and reduced (GSH) form
HAD	Hydroxyacyl-CoA dehydrogenase
HR	Heart rate
IMTG	Intramyocellular triglycerides
IVGTT	Intravenous glucose tolerance test
LT4	Lactate threshold, 4 mmol l^{-1}
mRNA	Messenger ribonucleic acid
mTOR	Mammalian target of rapamycin
OGTT	Oral glucose tolerance test
OXPHOS	Oxidative phosphorylation
PGC-1α	peroxisome-proliferator-activated receptor γ coactivator 1- α
RER	Respiratory exchange ratio
RM	Repetition maximum
ROS	Radical oxygen species
RTD	Rate of torque development
T2D	Type 2 diabetes mellitus
TT40	Time trial, 40 min
TTE-VO_2max	Time to exhaustion during VO_2max test

1 INTRODUCTION

Physical inactivity has been identified as one of the greatest public health problems in our time. Physical activity is defined as bodily movement produced by skeletal muscle that requires energy expenditure. Increasing physical activity brings many health benefits regarding cardiovascular disease, diabetes, cancer, hypertension, obesity, depression and osteoporosis (147). For these reasons, the American College of Sports Medicine (ACSM) has minimum recommendations regarding physical activity. Adults should perform at least 150 minutes per week of moderate intensity or 75 minutes per week of vigorous intensity cardio respiratory exercise training. On two to three days per week resistance exercise should also be performed (55). Meeting these recommendations with either resistance or aerobic exercise training is associated with a lower risk of diabetes of 34% or 52%, respectively (60).

Unfortunately, most individuals fail to meet these recommendations. At least partly because of that, insulin resistance and T2D are widespread diseases and major problems, both on individual and socioeconomic levels. Between 1980 and 2008, Type 2 diabetics increased across the world from 153 to 347 million (43). By the year 2050 diabetes prevalence is estimated to be as high as 33% in the U.S. population (25). An especially vulnerable group is the elderly. About one-third of the U.S. population over 60 years has diabetes, which is almost twice the proportion compared to middle-aged adults. Of these, approximately half are undiagnosed and an additional third have pre-diabetes (40).

However, exercise is not merely a tool for disease prevention; it is also the way to increase performance. It is well known that improved performance, up to a certain level, and health go hand-in-hand. For example, well trained individuals tend to be highly insulin sensitive (118) and there is a negative association between exercise capacity and mortality (83).

In a traditional view, depending on factors like intensity, duration, initial training status and genetic disposition, endurance exercise training results in improved aerobic capacity (68), while strength training results in increased strength and hypertrophy (136). However, the effects of the different exercise modes seem to be more intertwined and dependent upon initial training status. Effects previously considered specific for one mode, may in fact emerge from another. As an example, during some conditions, strength training can improve cycling economy (119). Similarities in the outcome between different exercise modes are not exclusive for performance; insulin sensitivity also benefits from both endurance and strength training (60). The health benefits of strength training have not been acknowledged for long; not until 1990 did ACSM add strength training to their recommendations on physical activity.

1.1 MITOCHONDRIA

Oxidative phosphorylation is a process that occurs in the mitochondria in which nutrients are metabolized while oxygen is consumed. The purpose of oxidative phosphorylation is to transform energy from nutrients into adenosine triphophate (ATP), the energy currency of the cell. The process is carried out through the metabolic pathways such as the citric acid cycle,

beta oxidation and the electron transport chain (ETC) (Figure 1). The ETC consists of five complexes across the inner membrane of the mitochondria. In a series of reduction and oxidation reactions, electrons are transported across the complexes. These reactions release energy that enables the transport of protons from the matrix to the inter-membrane space, which builds up a proton gradient across the membrane. The gradient then drives ATP synthase to resynthesize ATP from adenosine diphosphate (ADP). The redistribution of electrically charged protons creates an electrochemical gradient across the inner membrane.

The very central role of mitochondria in cell metabolism makes mitochondria a key player in several body functions and health issues. Mitochondrial density and function have been associated with cardiovascular disease (104), sarcopenia (106), insulin resistance and T2D (135), aging (109) and aerobic capacity (75, 148).

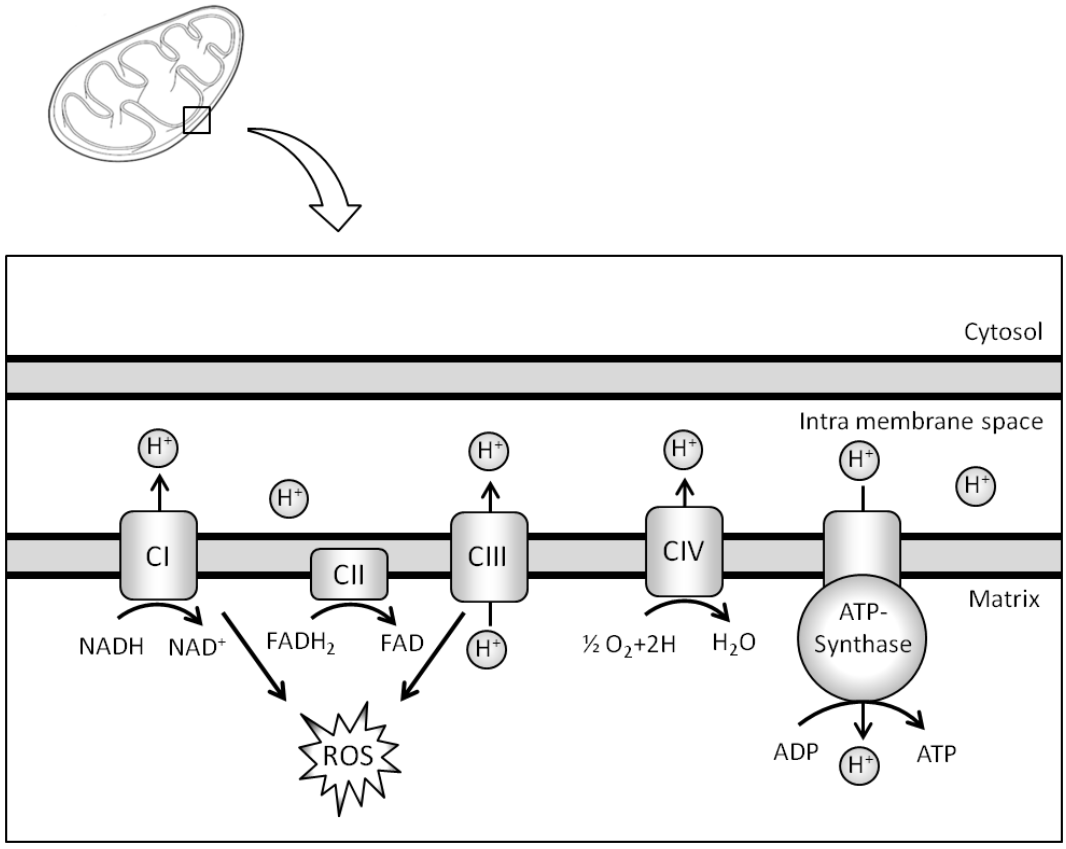

Figure 1*. The electron transport chain across the mitochondrial inner membrane. CI-IV, complex I-IV; H, hydrogen; ROS, reactive oxygen species.*

1.1.1 Reactive oxygen species (ROS)

Mitochondria are not merely energy transducers; they also produce ROS as a byproduct of oxidative phosphorylation. ROS are highly reactive compounds that can oxidize and react with different cell compartments. Endogenous and exogenous antioxidants neutralize ROS

and together they coexist in a balance referred to as the redox environment. The redox environment is affected by any changes in the presence of either ROS or antioxidants, e.g. through antioxidant supplementation or exercise. Since ROS are highly reactive molecules with the potential to damage cell structures, they have for several decades been viewed solely as something negative. More recently, research has identified ROS as a regulator of cell signaling, which suggests a more complex role (111).

Mitochondria are a major source of ROS (9). In diabetes, they seem to be responsible for the majority of excess chronic ROS production (102, 103). In the mitochondria, the main sites of ROS production are the ETC complexes I and III (13). Mitochondrial ROS formation is closely related to the electrochemical gradient across the inner membrane. ROS formation is low until the gradient rises and then it becomes sensitive to small changes (85). High substrate availability increases the electron pressure in the ETC, which increases the electrochemical proton gradient. This makes ROS production sensitive to the substrate availability of the cell. For unknown reasons, mitochondria from Type IIB fibers seem to produce more ROS than those from Type I fibers (8). Muscle contraction, and thus exercise, are well known initiators of ROS production. However, observations from experiments in our lab (unpublished) and others (45, 81) show that mitochondria produce more ROS in state 4 (basal) respiration compared to active state 3 (maximal ADP stimulated) respiration. In addition, mitochondrial ROS production during contraction seems to be much lower than previously estimated (111). This shows that the major part of exercise induced ROS production probably emerges from sources other than the mitochondria, e.g. nicotinamide adenine dinucleotide phosphate (NADPH) oxidase and xanthine oxidase (111).

For several decades ROS production during exercise was seen as a negative byproduct that interfered with performance and recovery. This led to numerous studies investigating the beneficial effects of antioxidant supplementation in combination with exercise. Generally, the studies found reduced markers of oxidative stress. However, this did not coincide with any improvements in performance or recovery (63). More recently, several studies have shed new light on the role of ROS. Suppression of ROS by antioxidant supplementation seems to blunt the response to exercise training. Oral administration of antioxidants (vitamins C and E) hampered training effects on maximal running time in rats (58). In humans VO_2max, insulin sensitivity, transcription of peroxisome-proliferator-activated receptor γ coactivator 1- α (PGC-1α), peroxisome-proliferator-activated receptor γ (PPARγ) and endogenous antioxidants were hampered (58, 116). Thus, ROS seems to be an important factor for cell signaling and mediating exercise adaptation (111).

The ability to interfere with signaling pathways makes ROS a potential candidate for the development of insulin resistance. Increased ROS formation causes inhibiting phosphorylations of the insulin receptor substrate 1 (IRS-1), an intermediate of the insulin signaling pathway (103). The ability of ROS to induce insulin resistance in some conditions and yet be an essential part of exercise induced insulin sensitivity (116) is clearly a paradox. An explanation for this might be the duration of ROS exposure. Chronic elevated levels seem

to be harmful while short intense exposure, e.g. following exercise, seems to activate cell signals. Another possible explanation is the source of ROS production, e.g. mitochondria, NADPH oxidase or xanthine oxidase (111). As mentioned previously, mitochondria seem to be responsible for the elevated ROS levels in diabetics, while during contractions, other sources seem to dominate (111).

1.1.2 Aerobic capacity

Aerobic capacity is measured as the maximal oxygen uptake, VO_2max. The major physiological limitation for VO_2max is O_2 delivery to the muscle. The main cause for changes in VO_2max following training or long-term immobilization is changes in stroke volume (15). Although mitochondrial volume correlates strongly with VO_2max (70, 148), changes in mitochondria only correspond with minor changes in VO_2max (15). Even so, mitochondrial volume correlates with performance (75) and responds well to training (137). Increased mitochondrial density affects performance independent of VO_2max by maintaining cellular homeostasis at higher work rates relative to VO_2max, which will improve lactate threshold. Increased mitochondrial density also improves the ability to oxidize fat to preserve glycogen stores (69). It is therefore of great interest for the athlete to find new strategies to increase mitochondrial biogenesis and thereby performance.

1.1.3 PGC-1α

PGC-1α is a major regulator of mitochondrial biogenesis and plays an important role in cell metabolism. When activated, PGC-1α binds to and co-activates several nuclear transcription factors, including PPARγ, nuclear respiratory factors (NRF-1 and -2) and the mitochondrial transcription factor A (Tfam) (57). By doing so, the transcription of several different genes are induced and proteins are translated, including PGC-1α itself (20).

Exercise is a potent up-regulator of PGC-1α (20). The activation of PGC-1α following exercise is initiated by several mechanisms. Ca^{2+} release during muscle contraction phosphorylates calcium/calmodulin-dependent protein kinase type IV (CaMKIV) activates cAMP response element-binding protein (CREB), a potent activator of PGC-1α (155). Intracellular energy stress is another activator. An increase in the adenosine monophosphate (AMP)/ATP ratio activates AMP-activated protein kinase (AMPK) which triggers several pathways directed to increase ATP level. One of these pathways includes PGC-1α (76). PGC-1α activation seems also to be sensitive to the redox environment. Exposure of cultured myotubes with ROS caused an induction of PGC-1α. The addition of the antioxidant N-acetylcystein then inhibited the up-regulation (74). *In vivo,* following four weeks of exercise training with or without vitamin E and C supplementation, the exercise induced expression of PGC-1α was blunted with antioxidants (116). Another well-known activator of PGC-1α is p38 mitogen-activated protein kinases (p38 MAPK) (20). Contraction induced activation of p38 MAPK seem to be dependent on ROS production (159). Considering its strong expression in muscle tissue and the multiple functions of mitochondria, PGC-1α has received a lot of attention in the context of performance and health (35).

In the elderly, however, training seems to have a lower effect on PGC-1α protein content (88). This may be due to a generally low content in the elderly (79) or impairment in AMPK activation (113). In either case, over expression of PGC-1α in skeletal muscle of aged mice improved oxidative capacity, suppressed mitochondrial degradation, and prevented muscle atrophy (149).

Seemingly, there are many reasons to investigate PGC-1α in several different settings and populations. Although much research is performed, more is needed regarding the regulation of PGC-1α and the consequences from it.

1.2 TRAIN LOW

Availability of endogenous or exogenous carbohydrates is crucial for performance at submaximal or intermittent intensity, with duration over approximately 90 minutes. Based on that, the traditional recommendation for athletes has been a high carbohydrate intake, even during training periods. However, more recent research has questioned this strategy. Studies show little benefit of training with high carbohydrate intake (29). Ingestion of carbohydrates during exercise seems to blunt expression of genes involved in FA metabolism rather than stimulate those involved in carbohydrate metabolism (37). Instead, it has been suggested that training with low carbohydrate availability shifts substrate utilization towards FA, which will preserve glycogen stores and thereby increase performance. Mitochondrial density is associated with fat oxidation (69) and it is therefore possible that increased fat utilization may stimulate mitochondrial biogenesis.

Exercise increases gene expression of several metabolic genes that promote endurance adaptations. Depending on several factors, like exercise mode and substrate availability, the increase differs in size and duration (65). One of these genes, PGC-1α, has been linked to carbohydrate availability. As mentioned previously, PGC-1α is activated by AMPK and p38 MAPK, which are activated by carbohydrate-restricted exercise (39, 157). Increased AMPK activation following low glycogen exercise may be explained by increased AMP/ATP ratio due to low substrate availability. Another explanation is a quite recently discovered glycogen binding site on the AMPK β subunit (95). Glycogen inhibits AMPK by binding to it. When glycogen is metabolized, AMPK is released and available for activation. In addition, when restricting carbohydrate intake during recovery from glycogen depleting exercise, PGC-1α gene expression was prolonged (110). Therefore, PGC-1α expression is most likely affected by the glycogen levels when performing exercise.

A groundbreaking study in this area was performed by Hansson and colleagues (61). They performed a study in which the subjects trained one leg with normal glycogen levels, and the other with half of the sessions performed in a reduced glycogen state. Exercise performance and CS activity improved profoundly in the glycogen reduced leg compared to the normal leg. This study was followed by training studies using a whole body approach, with subjects training twice every other day or once every day. Although these studies found improvements in the mitochondrial markers citrate synthase (CS), hydroxyacyl-CoA dehydrogenase (HAD)

and succinate dehydrogenase (SDH) (98, 157), none could confirm an improved performance. A common feature for these studies is a relatively small difference in glycogen between groups (98, 157). It is therefore difficult to interpret if the mitochondrial biogenesis is related to glycogen, or something else. Train low is a promising concept that needs further investigation; to pinpoint the cause of potential effects, studies with clear differences in glycogen levels are needed.

1.3 CONCURRENT EXERCISE

The combination of strength and endurance training is often referred to as concurrent training. Although there are gaps to fill, the positive effect of adding strength to endurance training on endurance performance has been extensively investigated (119). Mainly, there seems to be a performance enhancing effect by improved exercise economy, possibly mediated by improved strength and power, altered neuromuscular function, and muscle fiber type switching towards a more enduring composition. There are discrepancies regarding the effect on power output and velocity at the lactate threshold (119). Regarding mitochondrial biogenesis, the majority of studies show no benefit when strength and endurance sessions are separated by several hours or days in subjects with low or moderate training levels (19, 22, 64).

However, when strength and endurance training are performed together in a single session, the results are contradictory. Sale and colleagues showed an increased CS activity (26%) in the group performing concurrent exercise compared to no effect in endurance training only (123). A second study from Sale and colleagues found a similar increase in both groups (124). In contrast, Nelson and colleagues found a profound blunting effect on both CS activity and VO_2max from concurrent exercise training. A possible explanation for this may be the load of the strength training. Nelson used a much higher load (3 x 6 repetition maximum (RM)) compared to the studies by Sale (6 x 15–20 RM). In addition, Nelson et al had the subjects perform strength training prior to endurance exercise, opposite to Sale et al. All three studies were performed in recreationally active subjects.

In an acute crossover study performed at our laboratory, PGC-1α mRNA was vastly increased following concurrent exercise. Recreationally active subjects performed an exercise session of cycling followed by either rest or a strength training session (60–70% 1RM, <15 reps). After the concurrent exercise session, mRNA expression of PGC-1α was about twofold higher compared to endurance exercise alone (146). The increased gene expression might be mediated by increased mammalian target of rapamycin (mTOR) phosphorylation (146). mTOR seems important for PGC-1α gene expression; when blocking mTOR with rapamycin in skeletal muscle tissue, PGC-1α gene expression decreased (41).

It seems that concurrent exercise has the potential to both increase and decrease mitochondrial biogenesis depending on exercise set up. It is therefore important to find out what factors decide the outcome and identify the optimal strategy. It is also important to carry

out the research in a trained population that is in the most need of alternative training strategies.

1.4 RESISTANCE TRAINING IN THE ELDERLY

A negative consequence of aging is a decrease in aerobic capacity. The most widely used measure of aerobic capacity, VO_2max, starts to decline at about 30 years. After 40 years it declines with about 10% per decade and after 70 years the decline is even more pronounced (51). The major factor explaining the age-related decline in VO_2max is most likely a decline in maximal heart rate, which is not related to physical inactivity (32). Other factors such as reduction in stroke volume, total blood volume, and muscle O_2 extraction may also contribute to the age-related decrease in VO_2max. However, when normalized to fat-free mass (FFM), these parameters seem to be influenced by physical inactivity rather than the aging process itself (18, 32).

Mitochondrial density and maximal respiration are also reduced in the elderly, probably to a large extent caused by physical inactivity (109). Mitochondrial dysfunction has also been implicated as a central part of the ageing process (109). According to the mitochondrial theory of aging, increased mitochondrial production of reactive oxygen species results in DNA mutations, which initiates apoptosis and leads to reduced mitochondrial density and function (109). In addition, mitochondrial dysfunction has been suggested to play a central role in several age-related health impairments like sarcopenia (106), insulin resistance, T2D (135) and cardiovascular disease (104). Therefore, it is highly desirable to find strategies to counteract/prevent mitochondrial dysfunction and thereby the associated health impairments.

A well-known strategy to improve VO_2max and induce mitochondrial biogenesis in both young and the elderly is endurance exercise (68, 96). The effect of resistance exercise on these parameters is less investigated, especially in aged subjects. Since VO_2max and mitochondrial function is reduced in the elderly, especially individuals with low physical activity levels, resistance training may have a more beneficial effect compared to that in the young. Some, but not all (31), studies show a positive effect on VO_2max (52, 152). Regarding mitochondrial density, several studies have investigated the effect but the results are conflicting (11, 47, 52, 139).

1.5 INSULIN SENSITIVITY

The ability of the body to lower blood glucose levels in response to insulin is referred to as whole body insulin sensitivity. Physical inactivity, obesity and a poor diet may reduce the ability to dispose glucose from the blood. If glucose uptake is reduced below a certain level, impaired glucose tolerance develops, which is also referred to as pre-diabetes. Skeletal muscle accounts for approximately 80% of glucose disposal, which makes the muscle tissue a major player for regulating blood glucose levels. The first stage of developing T2D involves a reduced ability of muscular tissue to increase glucose uptake in response to insulin. To maintain blood glucose homeostasis, the body responds by increasing insulin excretion from pancreatic beta cells. As the insulin resistance progresses, insulin excretion amplifies. As

endogenous insulin becomes unable to maintain glucose homeostasis, diabetes develops. In the last stage of diabetes, beta cells become exhausted and the pancreas loses its ability to produce insulin.

The interference between carbohydrate and fat uptake and oxidation has been investigated for many decades. In 1963 Randle and colleagues showed that fatty acids (FA) impaired insulin-mediated glucose uptake by inhibiting pyruvate dehydrogenase (112). Since then several studies have connected insulin resistance to fat metabolism. Increased lipolysis, plasma FA or intra myocellular triglycerides (IMTG) are common features of many conditions associated with reduced insulin sensitivity, e.g. obesity, T2D, lipid infusion (10), high-fat diet (10, 78) and starvation (78). One theory suggests that an accumulation of IMTG causes insulin resistance. Much research has been performed in this area and there is a strong connection between increased IMTG and insulin resistance (143). The impairing effect seems to be mediated by toxic lipid intermediates, e.g. diacyl glycerols (DAG) and ceramides, that interfere with insulin signaling on several levels (143). Although the theory is supported by mounting evidence, it seems the connection is quite complex. Muscular contents of IMTG, DAG and ceramides are also increased in highly insulin sensitive endurance athletes, a contradiction referred to as "the athlete's paradox" (3).

In addition to having an increased level of IMTG, several studies show that individuals with insulin resistance or T2D have reduced mitochondrial content and function (143). Based on these observations it has been suggested that inherited or acquired mitochondrial dysfunction compromises the ability to oxidize fat. This may in turn cause IMTG accumulation and insulin resistance (125). Despite a large body of evidence, the theory has been questioned. Studies finding mitochondrial impairments in diabetics and insulin-resistant individuals have been criticized for not considering physical activity (121). When comparing healthy controls, pre-diabetics and longstanding T2D individuals, mitochondrial dysfunction was only found in the latter. This indicates that mitochondrial dysfunction develops during diabetes rather than precedes it. When the subjects then performed exercise training for a year, several of the mitochondrial impairments were reversed (144). In addition, muscles have a large spare oxidative capacity to use at high-intensity activities (12); the small reductions seen in insulin-resistant individuals are therefore not likely to compromise fat oxidation during low to moderate intensities in everyday living. Therefore, mitochondrial dysfunction is related to physical inactivity and likely not causative of insulin resistance.

Several studies have also contradicted the theory that mitochondrial dysfunction precedes insulin resistance and laid a platform for an alternative theory. Rodents fed a high-fat diet induce insulin resistance while increasing fat metabolism (142). In addition, inhibition of FA uptake into mitochondria prevents high-fat diet induced insulin resistance (86). It is possible that increased, instead of reduced, fat metabolism contributes to insulin resistance. Increased fat metabolism stimulates the production of ROS (7, 128) and there is a strong connection between ROS and insulin resistance (71, 103). Even more important, blocking mitochondrial

ROS production with antioxidants prevents high-fat diet induced insulin resistance (7, 24, 66) (Figure 2).

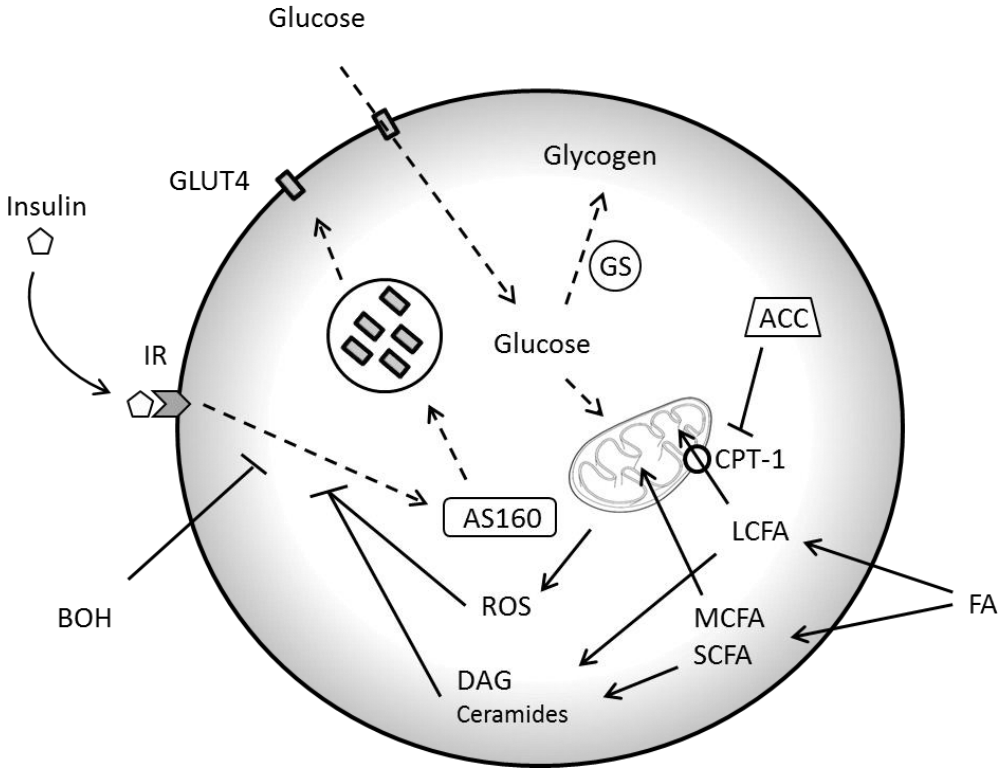

Figure 2. *Inhibition of Insulin stimulated glucose uptake by FA and ROS. FA, fatty acid; IR, insulin receptor; GLUT4, glucose transporter 4; GS, glycogen synthase; AS160, Akt substrate of 160 kDa; ROS, reactive oxygen species; DAG, diacyl glycerols; SCFA, short chain fatty acid; MCFA, medium chain fatty acid; LCFA, long chain fatty acid; CPT-1, Carnitine palmitoyltransferase I; ACC, Acetyl-CoA carboxylase; BOH, β-hydroxybutyrate.*

Although mitochondrial dysfunction may not cause insulin resistance, improving mitochondrial function through exercise may improve insulin resistance. Increasing mitochondrial density by exercise utilizes substrate oxidation towards fat (69). An improved ability to oxidize fat will make it easier to handle increased levels of fatty acids and may thereby prevent insulin resistance. In addition, it is well known that exercise training reduces oxidative stress and up-regulates anti-oxidative defenses (48, 105). An improved ability to chronically maintain the redox homeostasis may be one of the mechanisms behind improved insulin sensitivity following training. Endurance exercise has for long been recognized as an efficient method to prevent insulin resistance in both young and old (88). Resistance exercise has been proven to improve insulin sensitivity in younger adults (<65 years) (133) and the elderly with impaired glucose tolerance (73, 126). In healthy elderly (>65 years) studies are contradictory (44, 158) and there is need for further research.

PGC-1α may also play a role in insulin sensitivity. The finding that PGC-1α gene expression is reduced in diabetics (107) has led to the assumption that PGC-1α may be important for insulin sensitivity. However, genetically engineered mice contradict this hypothesis. When PGC-1α is over expressed, high-fat diet induced insulin resistance actually worsens (50). In contrast, exercising seems to be more insulin sensitizing in these mice compared to wild type mice (134). Although these findings show an involvement of PGC-1α in insulin sensitivity, its role is far from established.

1.5.1 Starvation

It is well established that short-term starvation in humans induces insulin resistance (93). Starvation may well be one of the earliest, if not the first, forms of insulin resistance in humans. Elucidation of the mechanisms involved in this form of insulin resistance could therefore have implications for understanding the development of insulin resistance under other conditions, such as obesity and T2D.

Within 24 hours of starvation, liver glycogen is depleted (101) and thereafter insulin resistance develops in peripheral tissues. The reduced insulin sensitivity is an important physiological response to prioritize glucose for the central nervous system. Since exercise acutely stimulates both insulin-dependent and insulin-independent muscle glucose uptake (53, 120), there is a potential danger that exercise during a hypoglycemic state may compromise metabolic homeostasis. The effect of acute exercise on insulin sensitivity during starvation is, however, unclear and further studies are required.

Starvation is associated with increased lipolysis, and increases in plasma FA and IMTG (132). As mentioned previously, increased lipid accumulation and metabolism induce insulin resistance, possibly mediated by ROS. In addition, starvation, or rather carbohydrate deprivation, induces production of lipid-derived ketone bodies. Since untreated T2D results in carbohydrate deprivation on a cellular level, increased ketone bodies is a common feature. Ketone bodies reduce insulin sensitivity (156) and it is possible they play a role in the development of insulin resistance (4).

Starvation seems to reduce mitochondrial respiration in humans (67) and reduce ROS production in mice (127). However, more research is needed to fully understand the adaptation of the mitochondria to food deprivation.

2 AIMS

The overall aim of this thesis is to provide increased knowledge of how different exercise strategies improve performance and insulin sensitivity. The mitochondria represent a central part of this thesis considering their key role in health and performance. To be more specific, the aims were:

- To examine the effects of endurance exercise with low muscle glycogen on markers of mitochondrial biogenesis.
- To examine the effect of concurrent strength and endurance training on markers of mitochondrial density and aerobic capacity.
- To examine the effect of exercise during starvation on insulin sensitivity.
- To examine the effects of resistance exercise training in the elderly on insulin sensitivity, as well as mitochondrial biogenesis, strength and aerobic capacity in the elderly.

3 MATERIAL AND METHODS

3.1 SUBJECTS

In Study I, the subjects were highly trained male cyclists with a history of competing at the national level. In Study II, the subjects were moderately trained male cyclists. In Study III, 9 recreationally active students participated in the study. In Study IV, 21 elderly (65-79 years) men and women with low physical activity level participated. One subject had a pacemaker (CON group), one asthma (RET group) and one was in an early stage of Parkinson's disease (RET group). The subjects with asthma and pacemaker were excluded from the submaximal cycling test. Subject characteristics are shown in table 1.

All subjects were informed about the possible risks and discomforts involved in the experiment prior to giving their written consent to participate in the study. The study design was approved by the Regional Ethics Committee of Stockholm, Sweden.

	n, female/male	Age (years)	Vo_2max (l min^{-1} kg^{-1})
Study I	0/10	27.8 ± 1.6	65 ± 1
Study II	19	34.7±1.2	56±1
Study III	4/5	23.2 ± 0.5	46 ± 2
Study IV	11/10	71.7 ± 0.8	-

Table 1. *Subject characteristics.*

3.2 INTERVENTION PROTOCOLS

3.2.1 Study I

Subjects participated in two experimental sessions in a crossover design with a high (normal glycogen, NG) or a low carbohydrate diet (low glycogen, LG). Both sessions included two exercise tests separated by about 14 h. The purpose of the first exercise session was to deplete muscle glycogen (depletion exercise) and the second exercise session to test the influence of low muscle glycogen on the exercise response (test exercise). The depletion exercise started with 45 min cycling at 75 % VO_2max followed by eight intervals at 88 % VO_2max (duty cycle 4 min exercise and 4 min active rest at 100 W), and ended with an additional 45 min at 70 % VO_2max. The test exercise included six intervals of 10 min cycling with 4 min active rest (100 W) between intervals. The first interval was at 72.5 % VO_2max after which the work rate was reduced 2.5 % during each interval (last interval 60 % VO_2max) (Fig. 3).

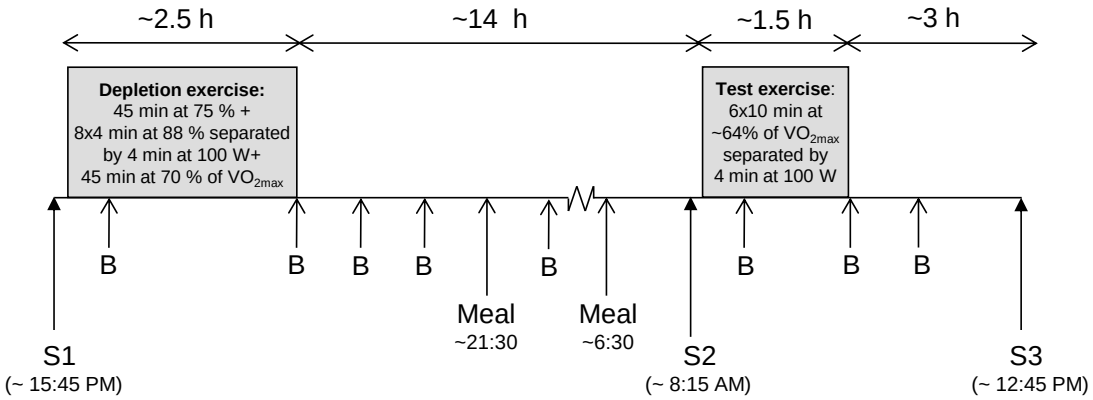

Figure 3. *Schematic illustration of the experimental design. B beverages containing CHO (NG normal glycogen) or only water (LG low glycogen). Meals contained either high (NG) or low CHO (LG). Beverages and meals post-exercise were separated by *1 h intervals and the beverages served during exercise were consumed ad libitum. Muscle biopsies and venous blood samples were obtained approximately 15 min before the depletion (S1) and test exercise (S2) as well as 3 h after the test exercise (S3).*

3.2.2 Study II

The subjects were divided into an endurance training group (E, n=10) or an endurance + strength training group (ES, n=9). They were instructed to continue their habitual cycle training but to exchange two ordinary training sessions per week with supervised laboratory training. The training consisted of 60 min of continuous cycling starting at a work rate corresponding to 90% of the mean power output during TT40 in the pre-test and then increasing to 95% throughout the intervention period. The strength training was performed in a leg press machine and consisted of a warm up set with 10 repetitions at 50% of 1 RM (determined in pre-test) followed by sets at 65, 70, 75, 75, 70, and 65% of 1 RM. Instead of resistance exercise the E group cycled for 2.5-4 min corresponding to an equal amount of energy expenditure (Figure 4).

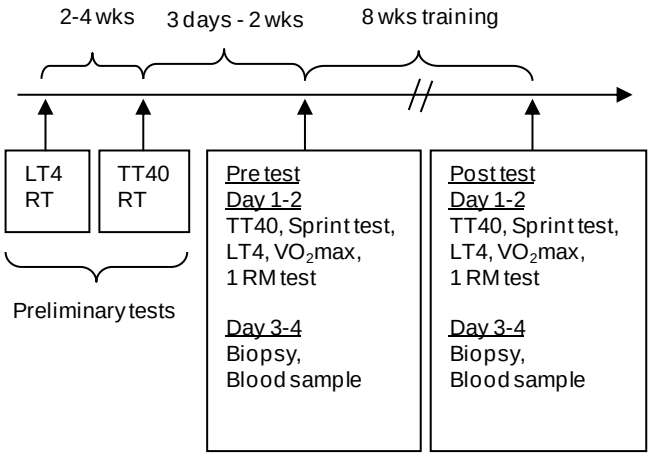

Figure 4. *Schematic illustration of the experimental protocol. LT4, 4 mmol l^{-1} lactate threshold test; RT, resistance training session, TT40, 40 min time trial; 1RM, one repetition maximum.*

3.2.3 Study III

Subjects participated in two experimental sessions separated by at least two weeks in a crossover design with randomized order. Both sessions included 75 h starvation with only water; in one of the sessions subjects performed an acute bout of exercise consisting of 5 x 10 min intervals separated by 2-4 min rest starting at 70% VO_2max. To avoid fatigue and be able to complete the exercise session, the intensity had to be gradually reduced to 50% VO_2max on the last interval (Fig. 5).

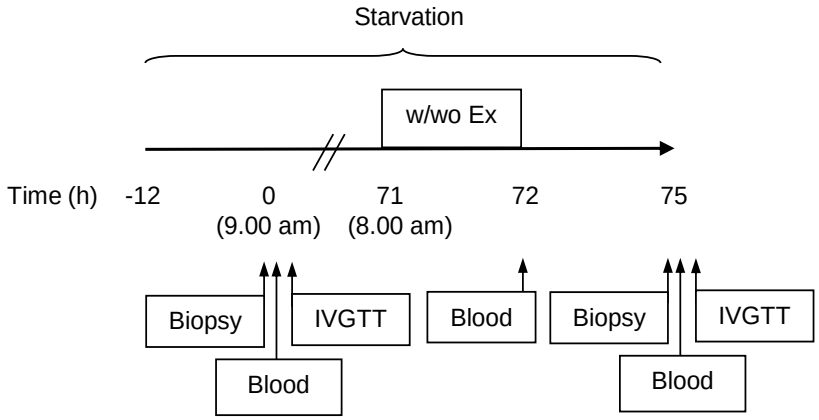

Figure 5. *Schematic illustration of the experimental protocol. w/wo Ex, with/without exercise; IVGTT, intravenous glucose tolerance test.*

3.2.4 Study IV

The subjects were divided into a resistance exercise training group (RET) or a control group (CON). The RET group performed 3 supervised sessions per week for 8 weeks. Each session started with a warm up and then the subjects performed exercises activating all major muscle groups (seated leg press, leg curl, leg extension, seated row, abdominal crunches, shoulder press and chest press). Each exercise was performed in 3 sets at 75-80 % of 1RM. If a subject was able to complete 12 repetitions in all sets of an exercise the load was increased ~5 % until next session.

3.3 PHYSIOLOGICAL TESTS

3.3.1 VO_2max (Study I, II and III)

The testing of VO_2max was performed on an ergometer (Monark 839E, Monark Exercise, Varberg, Sweden or SRM, Konigskamp, Germany) with a two stage incremental exercise protocol. The first part (3-5 min cycling at 5-6 submaximal intensities) was used to establish the relation between VO_2 and work rate (W) and to get a rough estimate of the work rate corresponding to VO_2max. After 3-5 min active rest, the work rate was increased rapidly until voluntary exhaustion with a protocol designed to elicit VO_2max after 7–8 min. Oxygen uptake was measured using an online system (Oxycon Pro, Erich Jaeger, Hoechberg, Germany or AMIS 2001; Inovision A/S Odense, Denmark) and VO_2max was defined as the highest recorded oxygen uptake during 40-60 consecutive seconds. The following criteria were used for attaining VO_2max: rating of perceived exertion (RPE) > 18, respiratory exchange ratio (RER) > 1.1, and a plateau of VO_2 with increased workload.

3.3.2 Lactate threshold (Study II)

The work rate corresponding to 4 mmol lactate per l blood (LT4) was determined during incremental submaximal exercise (the first part of the two stage VO_2max protocol). Capillary blood samples were collected from the finger tip immediately after each submaximal intensity and analyzed for lactate.

3.3.3 Time trial 40 min (TT40) and 30 s sprint tests (Study II)

The TT40 was performed on an ergometer (SRM, Konigskamp, Germany), preceded by warm up for 10 min. To standardize the test, subjects received information about the pacing strategy that they used during the preliminary test and were instructed to repeat this during the pre- and post-test. Subjects completed TT40 without any feedback other than the remaining time. Power output, cadence and heart rate were measured continuously. After the TT40 subjects had a total of 20 min of easy pedaling (~70W) followed by a 30 s maximal isokinetic sprint at 115 rpm in an “all out” fashion (Wingate test) with strong verbal encouragement. The sprint was performed seated and the subjects were informed of every 10 sec elapsed. Peak power was defined as the highest mean power output during 0.5 sec at any time during the sprint.

3.3.4 HR and RER during steady state cycling (Study IV)

In Study IV another measurement of endurance capacity was used considering the frailty of the subjects. Endurance capacity was established by measuring HR and RER during an exercise session on a cycle ergometer. The test consisted of two submaximal 4 min intervals with no rest between. The first interval was performed at a standard work rate (30 W) and the second at an individually based work rate (60-120 W). Mean HR and RER was calculated from the last minute of the intervals.

3.3.5 Strength and power (Study IV)

Knee extensor strength was measured as peak torque output during maximal voluntary isometric, concentric and eccentric right leg knee extension performed in a seated position using an isokinetic dynamometer (Isomed 2000, D&R, Hemau, Germany). The test was preceded by a warm up and several familiarization trials. During the test the subject performed four maximal voluntary eccentric and concentric knee extension actions (alternately) of the right leg at an angular velocity 30 deg • s-1 through a range of motion of 90 to 15° (0° = straight leg). After 4 minutes of rest four static measurements were made, at a knee angle of 65° (0° = straight leg). Torque signals were converted from analog to digital signals at 5 kHz using a CED 1401 data acquisition system and Signal software (Cambridge Electronic Design, Cambridge, UK). For each subject the test trial with the highest peak torque of the eccentric, concentric and static assessments, respectively, was used. Rate of torque development (RTD) was determined from the trial with maximal voluntary static contraction (MVC). RTD was derived as the mean slope of the torque-time curve (Δtorque/Δtime, unit Nm/s) over the time intervals 0-30 ms and 0-200 ms. Onset of contraction (time 0 ms) was defined as the time when knee extensor torque exceeded the baseline torque by 7.5 Nm.

3.3.6 1RM (Study II and IV)

The test started with a brief warm up set in the tested exercise. Thereafter the load was increased to near below an estimated 1RM. The subject performed 1 repetition and then the load was increased ~5 %. After sufficient recovery the procedure was repeated until failure. The highest load where 1 repetition could be performed was determined to be 1 RM.

3.3.7 Insulin sensitivity (Study III and IV)

In study III insulin sensitivity was measured with an intravenous glucose tolerance test (IVGTT) and in study IV with an oral glucose tolerance test (OGTT). Both tests were performed at least 48 h following exercise and 12 h following a meal. For the IVGTT venous cannulae were inserted into the antecubital vein of each arm. One arm was used for glucose infusion and the other for blood sampling. Basal samples were collected at 15 and 5 min prior to glucose infusion. Glucose was infused with a continuous flow over two minutes (0.3 g kg^{-1} body weight) after which blood samples were then collected at 1, 2, 3, 4, 5, 8, 10, 15, 20, 30, 40, 60 and 90 min following glucose infusion. The cannula was flushed frequently with saline

to avoid blood clotting. The samples were centrifuged at 1 500 g at 4°C for 10 min and plasma stored at -20°C for later analysis. Glucose tolerance was calculated as the area under the glucose curve above basal ($AUC_{glucose}$) and glucose disappearance rate (K_g) as the slope of the logarithmic glucose concentrations between 10 and 40 min. The method by galvin, SI_{galvin} (54), was used as a measure of whole body insulin sensitivity and was calculated as the ratio of Kg over the area under the insulin curve from 0 to 40 min above basal. Insulin release was measured as the area under the plotted curve above basal between 0 and 40 min ($AUC_{insulin\ 0-40\ min}$). Acute insulin response was calculated as the ratio between the areas under the curves for insulin and glucose above basal during the initial period (0 –10 min).

For the OGTT venous cannulae were inserted into the antecubital vein of one arm. Basal samples (4 ml) were collected at 15 min and immediately prior to glucose load (75 g glucose in a 250 g l^{-1} solution). Blood samples were then collected at 15, 30, 60, 90 and 120 min following glucose intake. The samples were centrifuged at 1 500 g at 4°C for 10 min and plasma stored at -20°C for later analysis. Area under the curve (AUC) for glucose, insulin and c-peptide was defined as the area under the curve above basal levels. Whole body insulin sensitivity was calculated with the Matsuda method (94) as $10{,}000*\sqrt{[(Glucose_{basal}*Insulin_{basal})*(Glucose_{mean}*Insulin_{mean}\ during\ OGTT)]}$.

3.4 ANALYTICAL METHODS

3.4.1 Blood sampling (Study I-IV)

Blood samples were collected from an anticubital vein (4 ml) and centrifuged at 1 500 g at 4°C for 10 min and plasma stored at -20°C for later analysis. To determine plasma FFA concentration a commercially available colorimetric enzymatic procedure (NEFA C test kit; Wako Chemicals GmbH, Neuss, Germany) was used (Study I and III). Venous plasma concentrations of Cortisol (Study II), Testosterone (Study II), Insulin (Study I, III and IV) and C-peptide (Study IV) were determined with commercially available ELISA kits (cortisol and testosterone, Calbiotech, CA, USA; insulin and c-peptide, Mercodia, Uppsala, Sweden). All plates were analyzed in a plate reader (Tecan infinite F200 pro, Männedorf, Switzerland). Glucose was analyzed in plasma and lactate in whole blood using an automated analyzer (Biosen 5140, EKF Diagnostics, Barleben, Germany).

3.4.2 β-hydroxybutyrate (BOH) (Study III)

The concentration of BOH in blood was analyzed with an enzymatic technique (55). Blood was mixed (2:1) with perchloric acid (0.65 M) and stored on ice. The sample was centrifuged at 3 000 g for 15 min, and the blood supernatant was stored at -80°C. One milliliter of supernatant was mixed with 0.1 ml K_2CO_3 (3.6 M) and incubated at 0°C for 5 min. The sample was centrifuged at 1 400 g for 30 s, the supernatant was transferred to another eppendorf tube, and pH was checked (pH 9.5). A 96-well plate was loaded with the samples and mixed with a reagent solution (4:1) consisting of glycine (330 mM), NAD (7.9 mM), and malate dehydrogenase (82 μg ml^{-1}). The absorbance was measured after 10 min at 340 nm with a plate reader (Tecan infinite F200 Pro; Tecan, Männedorf, Switzerland). Then, 5 μl of

sodium β-OH dehydrogenase (17 U ml^{-1}) was added to each well, and the absorbance was measured after 30 min when the reaction was finished. The concentration of BOH was calculated from the change in absorbance using a concentration curve.

3.4.3 Muscle sampling (Study I-IV)

Muscle biopsies were obtained from the middle portion of the vastus lateralis muscle using the percutaneous needle biopsy technique with suction (21) (Study I-III) or a Weil Blakesley conchotome (Wisex, Mölndal, Sweden) (Study IV). For histochemistry the samples were frozen in isopentane cooled to its freezing point in liquid nitrogen and stored at -80°C. For other analyses the samples were rapidly frozen in liquid nitrogen and stored at -80°C. Later the samples were freeze-dried, dissected free of blood and connective tissue and then homogenized in ice-cold medium specific for each analytical method.

3.4.4 Gene expression (Study I)

For mRNA analysis, total RNA was extracted from 2–5 mg freeze-dried muscle tissue using a Polytron PT 1600 E homogenizer (Kinematica, Lucerne, Switzerland) and a PureZOL RNA isolation kit according to the manufacturer's instructions (Bio-Rad Laboratories AB, Sundbyberg, Sweden). The yield and quality of extracted RNA were estimated by spectrometry and micro-gel electrophoresis (Experion, Bio-Rad). The 260/280 absorbance ratios were within 1.9–2.1 (in Tris–EDTA buffer, pH 8.0) and the RNA quality indicator values (RQI) were greater than 0.7. RNA (1 μg) was reverse transcribed to cDNA (20 μl) using the iScript cDNA synthesis kit (Bio-Rad). Real-time polymerase chain reaction (RT-PCR) was performed with an iCycler (Bio-Rad) in a mixture containing 12.5 μl 29 SYBR Green Supermix (Bio-Rad), 0.5 μl of both the forward and reverse primers (final concentrations 10 μM), and 11.5 μl template cDNA. All reactions were performed in triplicate with GAPDH as reference gene (91). The melting curves of the PCR product showed only one peak, demonstrating specificity of the primers and absence of contamination. The cDNA concentration, annealing temperature and thermocycling conditions were optimized for each primer pair, and assay sensitivity was high for all PCR products (RSq [0.99, and efficiency [90 %). The comparative critical threshold (CT) method could therefore be used to calculate changes in mRNA levels.

3.4.5 Glutathione analysis (Study I)

Glutathione in reduced (GSH) and oxidized (GSSG) form were determined with the Bioxytech GSH/GSSG-412 assay (Oxis Research, Foster City, CA, USA). The freeze-dried muscle tissue was divided into two aliquots and homogenized using glass homogenizers in ice-cold buffer (80 μl mg^{-1}) containing (in mM): Tris buffer (10), EDTA (1), EGTA (1), Na-orthovanadate (2), Na-pyrophosphate (2), NaF (5) and protease inhibitor cocktail, with or without 1-methyl-2-vinylpyridinium trifluoromethanesulfonate (M2VP), a scavenger of reduced GSH. Following 5 min incubation with 1 % Triton X-100 (room temperature for M2VP aliquot and ice cold for the M2VP free aliquot), 5 % metaphosphoric acid was added and the aliquots were centrifuged at 13 000 g for 10 min. The supernatant (5 μl) was diluted

1:20 with homogenization buffer and 100 µl chromogen, glutathione reductase and NADPH was added, followed by spectrophotometric measurement of the change in absorbance at 412 nm over 3 min. Reduced GSH was calculated from the measurements of total GSH (without M2VP in homogenate) and GSSG (with M2VP in homogenate). GSSG values are expressed in GSH units, i.e. 1 GSSG = 2 GSH.

3.4.6 Citrate synthase (CS) and Hydroxyacyl-CoA dehydrogenase (HAD) enzyme activity (Study II)

For determination of enzyme activity, muscle samples were homogenized using a bullet blender in a buffer (150 µl mg^{-1}) with the following composition (in mM): 50 K_2HPO_4, 1 EDTA and 0.05% Triton X-100 adjusted to pH 7.4. The homogenate was centrifuged at 10 000 rpm for 10 min and the supernatant was collected and diluted x3. CS activity was measured in a reagent solution (in mM): 50 Tris-HCl, 0.2 DTNB and 0.1 acetyl-CoA. The reaction was initiated by adding oxaloacetate (7 mM) and the change in absorbance at 412 nm was measured spectrophotometrically at 25°C. HAD activity was measured in a reagent solution (in mM): 65 Triethanolamine HCL, 0.3 EDTA and 0.3 NADH adjusted to pH 7.0. The reaction was initiated by adding acetoacetyl coenzyme A (4 mM) and the change in absorbance at 340 nm was measured spectrophotometrically at 25°C.

3.4.7 Western blot (Study I-IV)

The samples were homogenized using a bullet blender (Bullet Blender 1.5, Next Advance, NY, USA) in ice-cold buffer (80 µl mg^{-1}) with the following composition (in mM): 2 HEPES, 1 EDTA, 5 EGTA, 10 $MgCl_2$, 50 β-glycerophosphate, 1% TritonX-100, 1 Na_3VO_4, 2 Dithiothreitol, 20 µg ml^{-1} Leupeptin, 50 µg ml-1 Aprotinin, 1% Phosphatase inhibitor cocktail (Sigma P-2850, St Louis, MO, USA), 40 µg $µl^{-1}$ PMSF. In Study I-III the homogenate was centrifuged to pellet the insoluble debris, centrifugation was not performed in Study IV to avoid the possibility of losing proteins of interest. Protein concentration was determined with the bicinchoninic acid assay (Pierce Biotechnology, Rockford, IL, USA) by measuring the absorbance at 560 nm with a plate reader (Tecan infinite F200 pro, Männedorf, Switzerland). The samples were diluted with Laemmli sample buffer (Bio-Rad Laboratories, Richmond, CA, USA) and homogenizing buffer (1:1) to a final protein concentration of 1.5 µg $µl^{-1}$ and heated to 95°C for 5 min to denature proteins. The diluted samples were stored at -20°C prior to analysis. The proteins of the diluted samples were separated by SDS-PAGE (Criterion cell gradient gels, Bio-Rad Laboratories) for 45 min at 300 V on ice and then transferred to polyvinylidine fluoride membranes (Bio-Rad Laboratories) for 3 h at 300 mA on ice. The amount of protein loaded to the membranes was kept constant for all samples and was verified by staining with MemCodeTM Reversible Protein Stain Kit (Pierce Biotechnology). After blocking for 1 h at room temperature in 5% non-fat milk, the membranes were incubated over night with primary antibodies (see specific study). This was followed by 1 h incubation with anti-rabbit or anti-mouse HRP (1:10 000) as secondary antibody. The antibodies were visualized by chemiluminescent detection on a Molecular

Imager ChemiDocTM XRS system and the bands were analyzed using Quantity One® version 4.6.3 software (Bio-Rad Laboratories).

3.4.8 Mitochondrial respiration and ROS emission (Study I and III)

Muscle samples (10-25 mg wet weight) were stored in an ice cold medium with the following composition (in mM): 2.8 CaK_2EGTA, 7.2 K_2EGTA, 5.8 Na_2ATP, 6.6 $MgCl_2$, 20 Taurine, 15 Na_2Phosphocreatine, 20 Imidazole, 0.5 Dithiothreitol and 50 MES adjusted to pH 7.1. The specimen was split into 2-5 mg fiber bundles and each bundle was mechanically separated using surgical needles into a network formation to expose fiber membranes to the surrounding medium. The bundles were incubated with saponin (50 µg ml^{-1}), washed twice and stored in a medium with the following composition (in mM): 0.5 EGTA, 3 $MgCl_2$, 60 K-lactobionate, 20 Taurine, 10 KH_2PO_4, 20 Hepes, 110 Sucrose and 1 g l^{-1} BSA adjusted to pH 7.1.

Mitochondrial respiration was measured with either a Clark-type electrode (Hansatech instruments, Kings Lynn, England) (study I) at 25°C or an Oroboros oxygraph (Oroboros Instruments, Innsbruck, Austria) (study III) at 37°C in the storage medium. Oxygen level in chamber was adjusted to 400 nmol ml^{-1} with H_2O_2 and 280 U ml^{-1} Catalase. To reduce muscle contractions 45 µM Benzyltoluene sulfonamide was added (108). For measurement protocol, see separate study.

The rate of mitochondrial H_2O_2 production was measured with Amplex red (Invitrogen, Eugene, OR, USA), which, in the presence of peroxidase enzyme, reacts with H_2O_2 and produces the red fluorescent compound Resorufin. Permeabilized fiber bundles were added to the measuring medium (in mM): Mannitol (225), Sucrose (75), Tris-base (10), K_2HPO_4 (10), EDTA (0.1), MgCl2 (0.08), 2 g l^{-1} BSA, 13.5 U ml^{-1} Horse radish peroxidase, 45 µM Benzyltoluene sulfonamide, 45 U ml^{-1} SOD and 2 µg ml^{-1} Oligomycin, adjusted to pH 7.1 and kept at 30 °C. The change in fluorescence was recorded (Hitachi fluorescence spectrophotometer f-2500 with magnetic stirrer, Tokyo, Japan). For measurement protocol, see separate study.

3.4.9 Glycogen (Study I and III)

Glycogen was analyzed in 1-2 mg of freeze-dried muscle according to the method previously described by Harris et al (62), which includes enzymatic hydrolysis of glycogen followed by enzymatic analysis of glucose.

3.4.10 Histochemistry (Study IV)

Serial 10 µm-thick cross sections were cut at -20°C using a cryostat (Leica CM1950; Leica Microsystems, Wetzlar, Germany). The sections were then mounted on glass slides and air dried at room temperature. The sections were stained for myofibrillar ATPase at pH 9.4 after preincubation at pH 4.36, 4.65 and 10.37 (27). To visualize capillaries, the cross-sections were stained by the amylase-PAS method (6). One region of the cross section without artifacts and no tendency to longitudinal cuts of the fiber were selected and analyzed in a light

microscope (Olympus BH-2, Olympus, Tokyo, Japan) using Leica software (Leica Qwin V3, Leica Microsystems, Wetzlar, Germany). Fibers were measured for cross sectional area (CSA), capillaries and classified as type I, IIA or IIX.

4 RESULTS

4.1 TRAIN LOW

In Study I, at the initiation of the test exercise, muscle glycogen was significantly lower in LG (27 %) compared to NG (74 %). FFA increased in LG with 112 % pre-test exercise and 290 % post-test exercise. FFA increased in LG with 112 % pre-test exercise and 290 % post-test exercise. Blood glucose was slightly reduced in both NG and LG, with no difference between conditions, and insulin was reduced in LG and slightly elevated in NG (Figure 6).

Although PGC-1α mRNA increased in both groups 3 h following test exercise, the expression was higher in LG compared to NG (8.1-fold and 2.5-fold, respectively) (Figure 7).

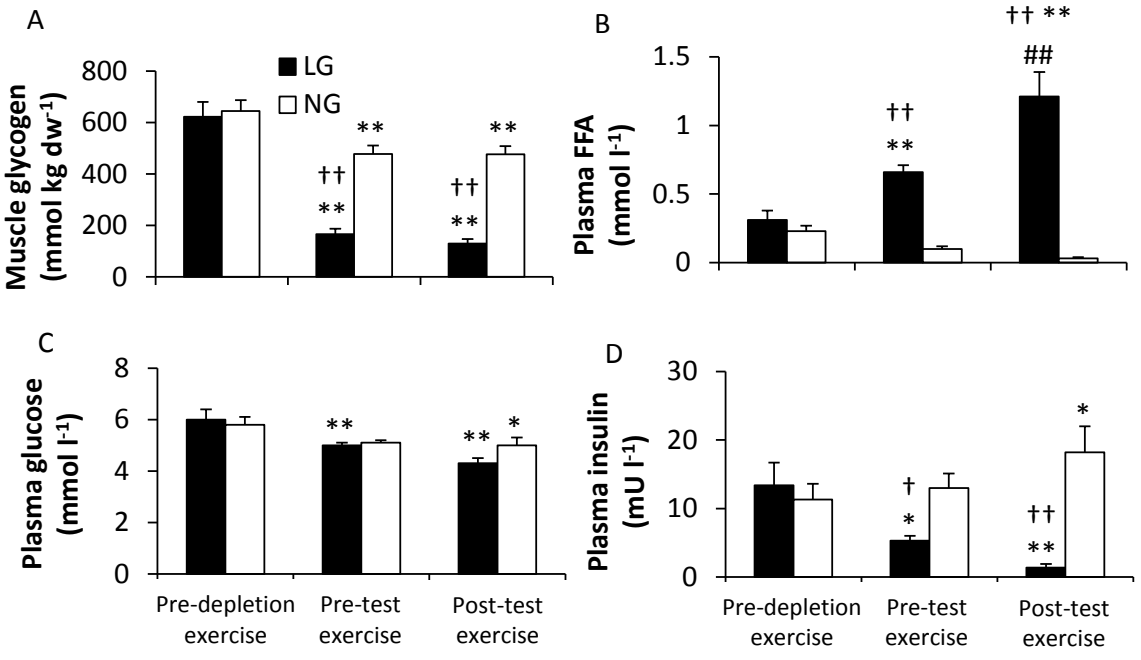

Figure 6. *Study I. Metabolic responses in (A) muscle glycogen, (B) plasma free fatty acids, (C) plasma glucose and (D) plasma insulin. LG, low glycogen; NG, normal glycogen; Values are reported as means ±SE (n=10). * P<0.05 and ** P<0.01 vs pre-depletion exercise; ## P<0.01 vs pre-test exercise; † P<0.05 and †† P<0.01 vs NG.*

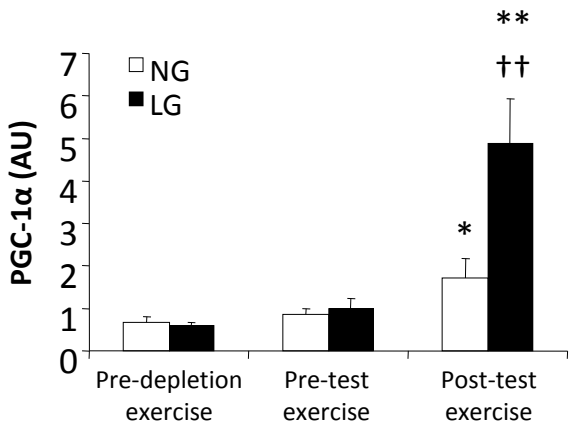

Figure 7. *Study I. Effect of exercise on mRNA levels of PGC-1α. LG, low glycogen; NG, normal glycogen; Values are expressed as means±SE (n=10). *P<0.05 and **P<0.01 vs Pre-depletion exercise; ††P<0.01 vs NG.*

4.2 CONCURRENT EXERCISE

In Study II, VO_2max increased similarly in both groups and time to exhaustion during the VO_2max test increased in ES (+10%). LT4 and mean power output during the 40 min time trial increased in E (+3% and +4%, respectively) (Table 2).

	E		ES	
	Pre	Post	Pre	Post
VO_2max (ml^{-1} min^{-1} kg^{-1})	55±1	58±1**	57±1	58±1*
TTE- VO_2max (s)	402±14	423±15	407±22	443±21**
LT4 (W)	287±11	295±11*	283±9	286±8
TT40 (W)	284±11	294±11*	282±10	290±10

Table 2. *Study II. Effect of training on performance and performance related variables. TTE-VO_{2max}, time to exhaustion during the VO_{2max} test; LT4, work rate corresponding to 4 mmol lactate per l blood; TT40, 40 min time trial. E, endurance training only (n=10); ES, endurance + strength training (n=9). Values are reported as means ± SE. Main effect of training: *; P < 0.05 vs. pre-training; **; P < 0.01 vs. pre-training.*

HAD and CS activity was unaffected by concurrent training (+3% and -1%, respectively) and endurance training (+11% and +9%, respectively) (Figure 8a and b). Training had no effect on the blood concentration of plasma testosterone or cortisol levels at rest (data not shown).

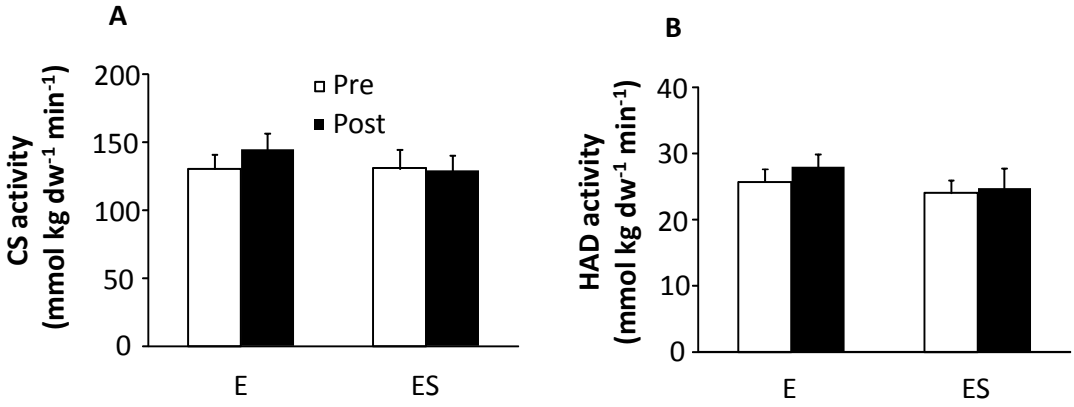

Figure 8. *Study II. Effect of 8 weeks of training on mitochondrial enzyme activities. E, endurance training only (n=10); ES, endurance + strength training (n=9). CS, citrate synthase; HAD, 3-hydroxyacyl-CoA dehydrogenase. Values are reported as means ±SE.*

4.3 RESISTANCE TRAINING IN ELDERLY

In Study IV, training resulted in hypertrophy as indicated by increased FFM (+1.4 kg), thigh circumference (3.3%) and thigh area (6.7%) in RET. However, fiber CSA did not increase (Table 3).

	RET		CON	
	Pre	Post	Pre	Post
FFM (kg)	51.0±2.3	52.4±2.1**	47.6±4.1	48.6±4.3
Thigh circumference (cm)	48.6±1.2	50.1±1.0***†	46.4±1.6	46.1±1.5
Thigh CSA (cm^2)	188±9	200±8***†	155±12	154±11
Fiber CSA (μm^2)				
Type I	5452±393	5567±362	4889±323	4807±354
Type IIa	4230±610#	4484±434#	4114±535#	3971±494#
Type IIx	3678±634#	3554±552#	3392±889#	2913±427#

Table 3. *Study IV. Physical characteristics before and after resistance exercise training. RET, resistance exercise training (n=12); CON, control (n=9); Fiber CSA: RET (n=10) and CON (n=7); FFM, fat free mass; CSA, cross-sectional area. Values are presented as mean±SEM. **p<0.01 vs pre; ***p<0.001 vs pre; † p<0.05 vs CON post; # p<0.05 type II (a and x) vs type I.*

Strength measured as torque and power as RTD increased following resistance exercise. Knee extensor strength improved in RET during isometric (+12%), concentric (+11%), and eccentric (+8%) contraction (Figure 9a). RTD during 0-30 ms of the peak MVC increased by 52%. RTD during 0-200 ms of peak MVC did not change significantly (Figure 9b). For each exercise the load during the training period increased significantly ($p<0.05$) as follows: seated leg press +30%, abdominal crunches +61%, chest press +24%, lat spread +38%, shoulder press +72%, seated row +58%, leg extension +19% and seated squats +41%. Signaling proteins related to muscle protein synthesis, Akt and mTOR, increased by 69% and 69%, respectively (Figure 10a and b).

Histological analysis showed a significant increase from resistance training in the amount of type IIa fibers (27 ± 7 %, $p<0.005$) and a strong trend for decreased amount of type IIx fibers (16 ± 18 %, $p=0.068$) (Figure 11a and b).

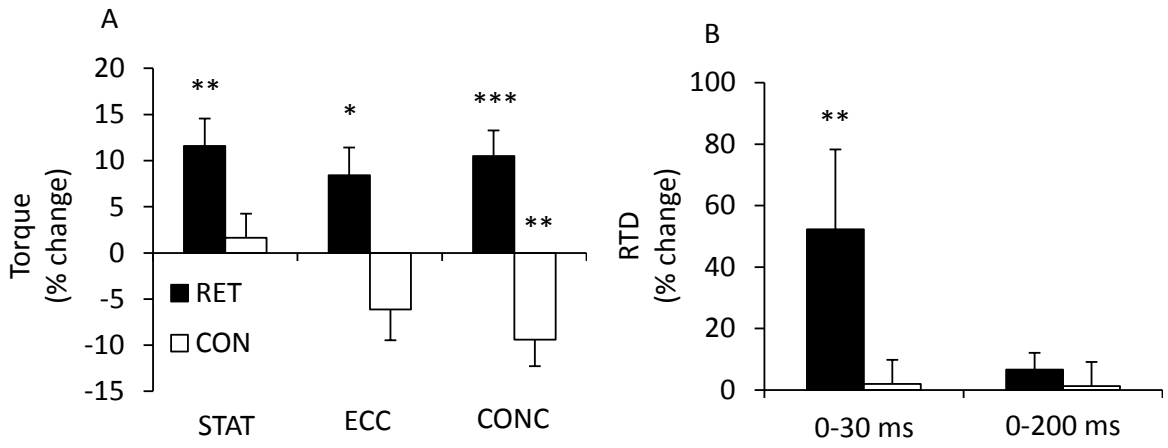

Figure 9. *Study IV. Changes following resistance exercise training in (A) eccentric, static and concentric MVC and in (B) RTD during 0-30 ms and 0-200 ms of the peak MVC. RET, resistance exercise training (n=12); CON, control (n=9); Values are presented as % ± SEM relative to basal values (mean±SEM). *p<0.05 vs pre; **p<0.01 vs pre, ***p<0.001 vs pre.*

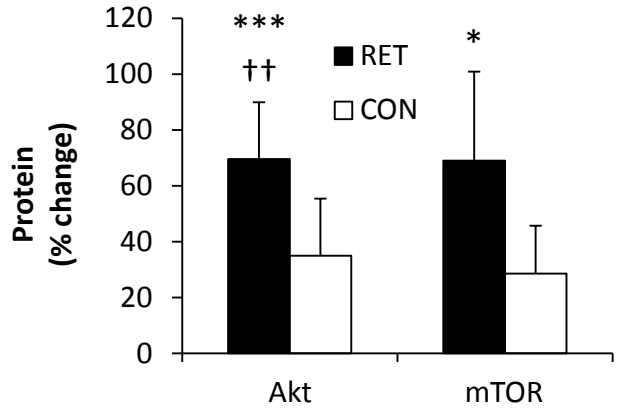

Figure 10. *Study IV. The effect of resistance exercise training on protein levels of total mTOR and Akt. RET, resistance exercise training (n=12); CON, control (n=9); Values are presented as % ± SEM relative to basal values (mean±SEM). *p<0.05 vs pre; ***p<0.001 vs pre; †† p<0.01 vs CON post.*

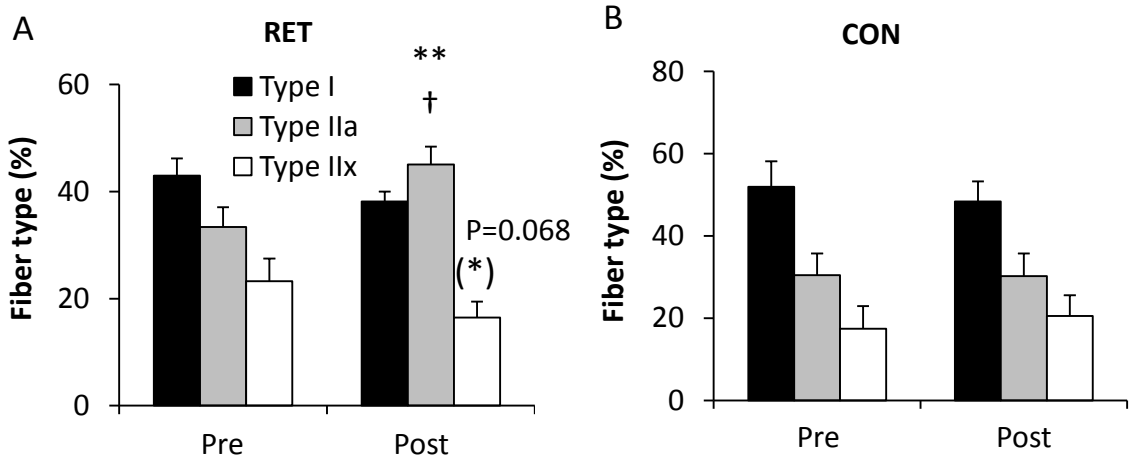

Figure 11. *Study IV. The effect of resistance exercise training on muscle fiber type composition in (A) RET, resistance exercise training (n=12) and (B) CON, control (n=9); Values are presented as means ± SEM. (*) strong trend vs pre; **p<0.01 vs pre; † p<0.05 vs CON post.*

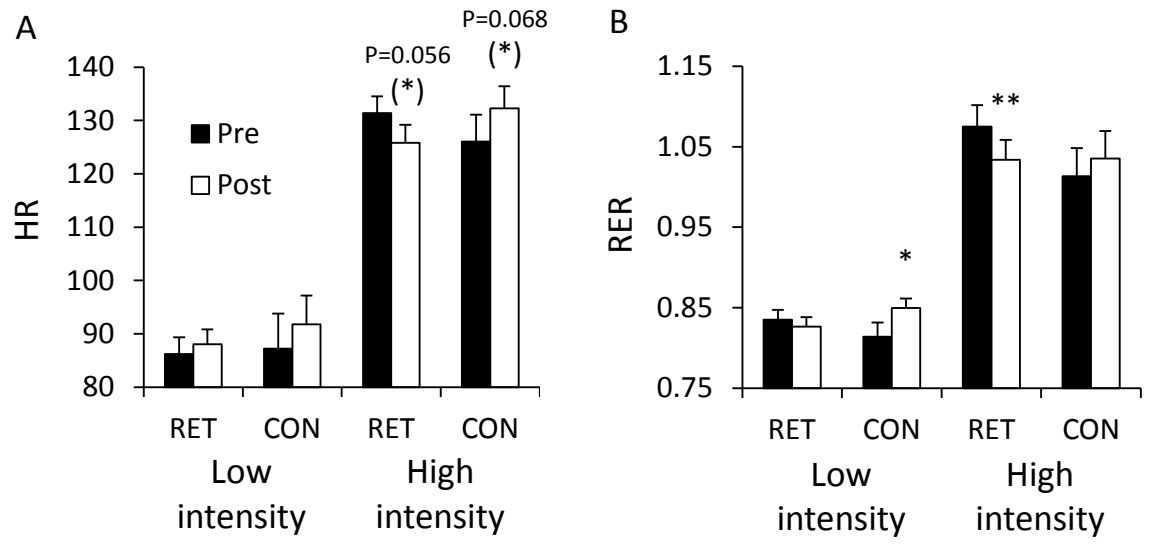

Figure 12. *Study IV. Cardio respiratory data pre and post resistance exercise training. (A) HR, heart rate and (B) RER, respiratory exchange ratio during low (30 W) and high (60-120 W) intensity steady state cycling. Two subjects were excluded from the submaximal cycling test due to asthma and pacemaker. RET, resistance exercise training (n=11); CON, control (n=8); Values are presented as mean±SE. (*) Strong trend vs pre; *p<0.05 vs pre.*

During cycling at the lowest intensity interval HR was unchanged in both groups (Fig. 12a), however, RER increased in CON (+4.1%) with no change in RET (Fig. 12b). At the highest intensity interval there were strong trends for a decrease in heart rate in RET (-4.7%, p=0.056) and an increase in CON (+4.6%, p=0.068). RER decreased in RET (-4.0%). Protein levels of well known markers for mitochondrial volume, OXPHOS complexes II, IV and CS, increased by 30, 99 and 30%, respectively (Fig. 13).

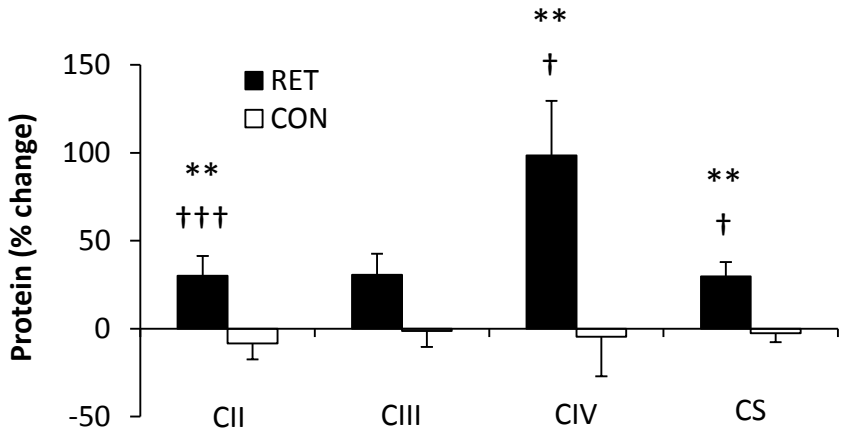

Figure 13. *Study IV. The effect of resistance exercise training on mitochondrial proteins OXPHOS complex II, III, IV and citrate synthase. RET, resistance exercise training (n=12) and (B) CON, control (n=9); Values are presented as % ± SEM relative to basal values (mean±SEM). **p<0.01 vs pre; † p<0.05 vs CON post; ††† p<0.001 vs CON post.*

4.4 STARVATION

In Study III, plasma levels of BOH increased 50-fold after starvation. Although BOH decreased immediately following exercise, it increased to the same levels as NE 3 h post exercise. FA increased 3.3-fold in NE and 4.1-fold in EX, with a significant difference between conditions. Starvation resulted in marked decreases in basal plasma glucose and insulin, without differences between groups Muscle glycogen was reduced in both conditions with a significantly stronger reduction in EX (Figure 14a-e).

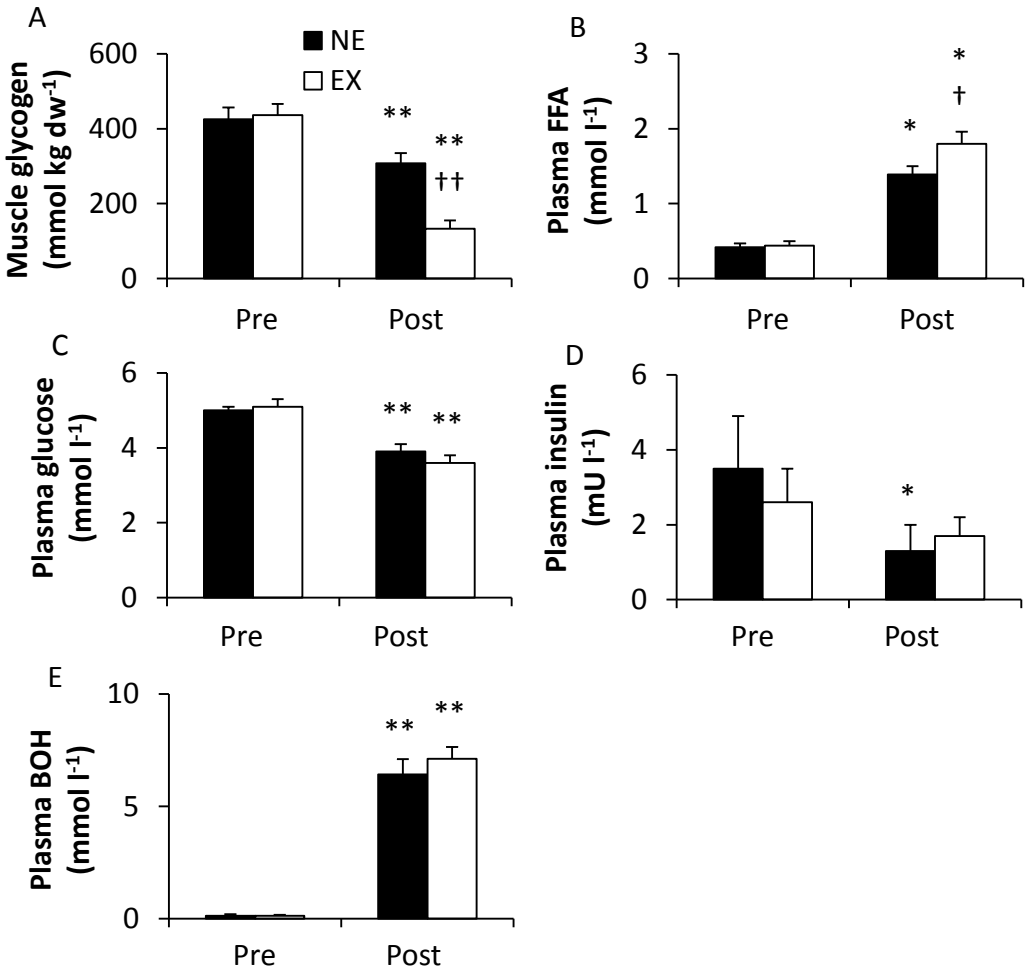

Figure 14. *Study III. Metabolic responses to starvation and exercise. NE, without exercise; EX, with exercise (n=9) 3 h prior to intravenous glucose tolerance test (IVGTT); FA, fatty acids. Muscles and blood samples were taken after an overnight fast prior to IVGTT (basal) and after 75 h of starvation. Values are reported as means ± SE. *P<0.05 and **P<0.001 vs. prestarvation; †P<0.05 and ††P<0.001 vs. NE.*

4.5 INSULIN SENSITIVITY

In Study III, during the IVGTT plasma glucose was increased between 30 and 90 min in both groups after starvation. However, the increase was significantly higher in NE (Figure 15a and b). From the IVGTT, K_g decreased by 65% in NE and 37% in EX, and $AUC_{glucose}$ increased by 250 and 181% in NE and EX, respectively. Although both parameters demonstrate reduced glucose tolerance in both groups, they show a higher glucose tolerance in EX. Whole body insulin sensitivity (SI_{galvin}) was reduced in NE (to 59%) after starvation, in EX it was unchanged and therefore significantly higher. Acute insulin response and $AUC_{insulin\ 0–40\ min}$ decreased by 30–35% in both conditions, indicating reduced insulin secretion following starvation (Table 4). Phosphorylated GS $Ser^{641/645}$ and AS160 Thr^{642} were increased by 20% and 138%, respectively, following starvation in NE (Figure 16a and b).

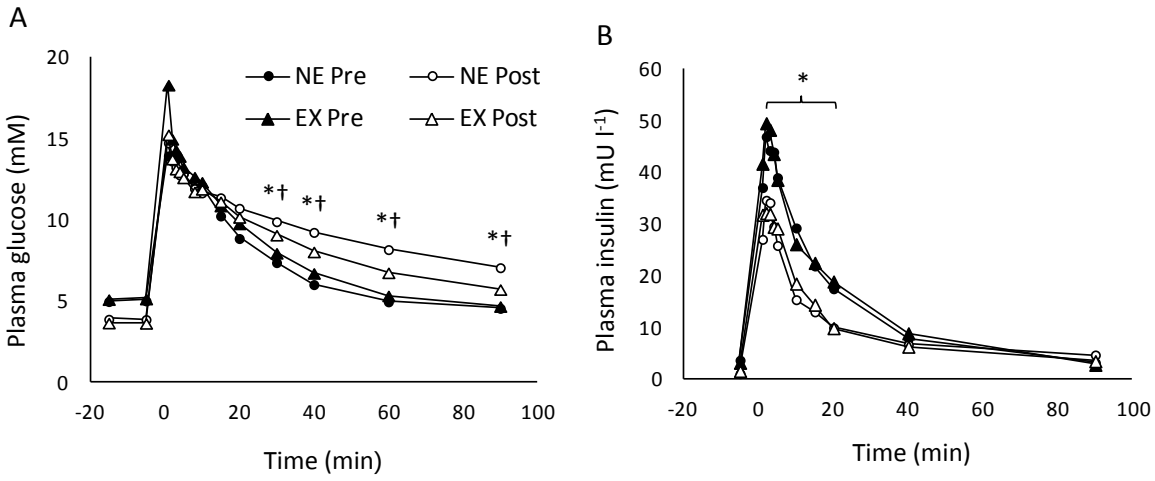

Figure 15. *Study III. Plasma glucose and insulin concentrations during IVGTT. Tests were performed before and after 75-h starvation with (EX) or without exercise (NE) (n = 9). (A) plasma glucose, (B) plasma insulin; For clarity only, mean values are shown. *P<0.05, post exercise (post) vs. pre exercise (pre); †P<0.05, EX post vs. NE post.*

	NE		EX	
	Before	After	Before	After
SI_{Galvin} (10^{-5} min^{-1} [mU min^{-1} l^{-1}]$^{-1}$)	1.2±0.4	1.1±0.3*	1.4±0.3	1.6±0.4†
K_g (10^{-2} min^{-1})	1.0±0.1	0.4±0.1**	0.9±0.1	0.6±0.1*†
$AUC_{glucose}$ (mmol min l^{-1})	193±15	483±23**	234±19	423±26**†
$AUC_{insulin\ 0–40\ min}$ (mU min l^{-1})	723±142	496±105**	759±139	502±101**

Table 4. *Study III. Metabolic responses to IVGTT. K_g, rate of glucose disappearance; $AUC_{glucose}$, area under plasma glucose curve above basal; SI_{galvin}, K_g $(AUC_{insulin\ 0–40\ min})^{-1}$; 1. Subjects (n=9) participated in 2 experimental sessions with 75 h of starvation. Values are reported as means ± SE. *P<0.05 and **P<0.001 vs. prestarvation; †P<0.05 vs. NE.*

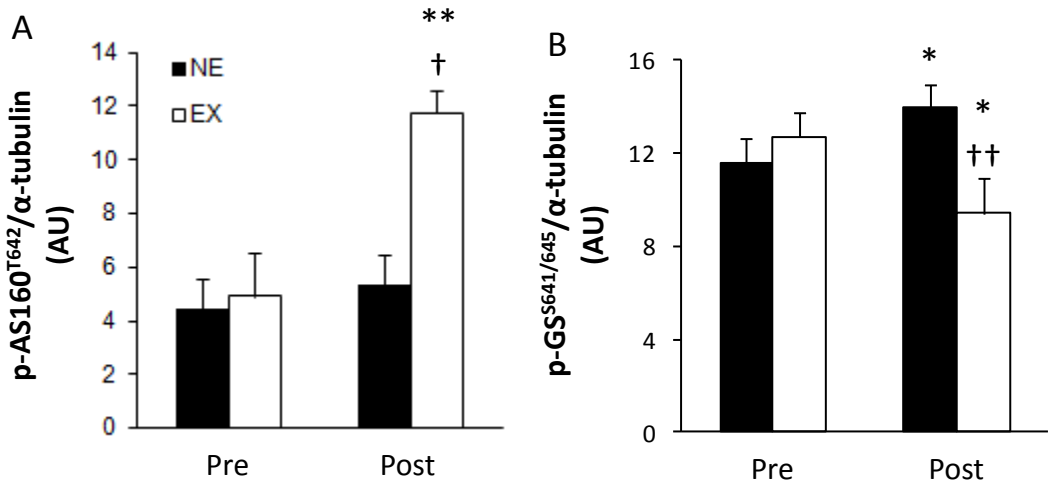

Figure 16. *Study III. Effects of starvation and exercise on regulatory proteins. Muscle biopsies were obtained before and after 75-h starvation with (EX) or without exercise (NE) (n = 8). Biopsies were taken prior to IVGTT. (A) Akt substrate of 160 kDa (AS160) Thr^{64}, (B) glycogen synthase (GS) Ser$^{641/645}$. Values are reported as means ± SE. *P< 0.05 and **P<0.001 vs. pre; †P<0.05 and ††P<0.001 vs. NE post.*

In Study IV, OGTT showed that glucose tolerance was improved after training in RET both when measured as $AUC_{glucose}$ (-21%) and plasma glucose 2 h post glucose intake ($Glu_{120\ min}$ -14%) (Figure 17a and b). Applying cut off levels recommended by WHO ($Glu_{120\ min}$ >7.8 mmol l^{-1}) (150), three individuals in the RET group were classified as having impaired glucose tolerance at baseline. However, following training plasma glucose was normalized in these three subjects. When the three subjects with impaired glucose tolerance were excluded, the improvements in $AUC_{glucose}$ and $Glu_{120\ min}$ were no longer significant. In the CON group one subject developed impaired glucose tolerance over the intervention period, but mean values of $AUC_{glucose}$ and $Glu_{120\ min}$ were not changed after the intervention period.

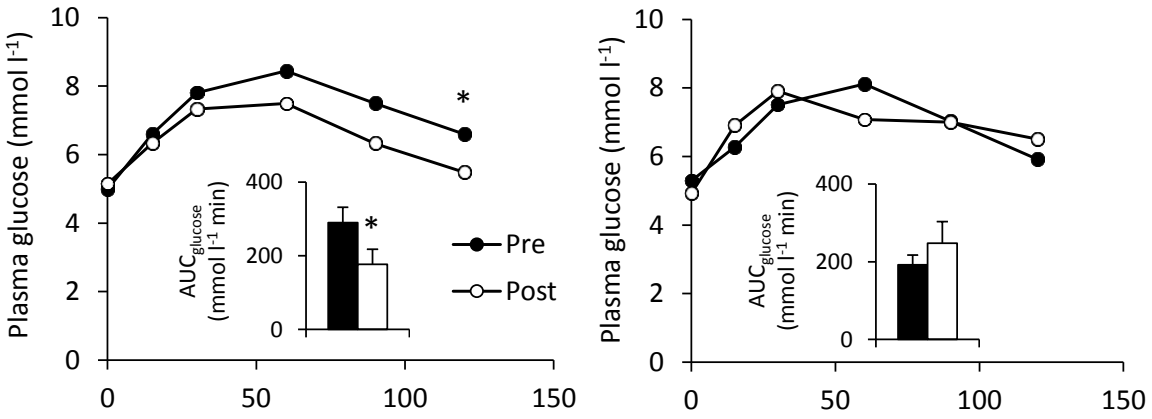

Figure 17. *Study IV. Response to OGTT in plasma glucose for (A) RET, resistance exercise training (n=12) and (B) CON, control (n=9); RET pre training values include 3 subjects with impaired glucose tolerance. Values are presented as means ± SE. *p<0.05 vs pre.*

4.6 MITOCHONDRIAL RESPIRATION AND FA METABOLISM

In study I, phosphorylated acetyl-CoA carboxylase (ACC) Ser^{79} did not change (Figure 18a). In study III, exercise during starvation lead to a significantly increase in phosphorylated ACC Ser^{79} (+24 %) (Figure 18c).

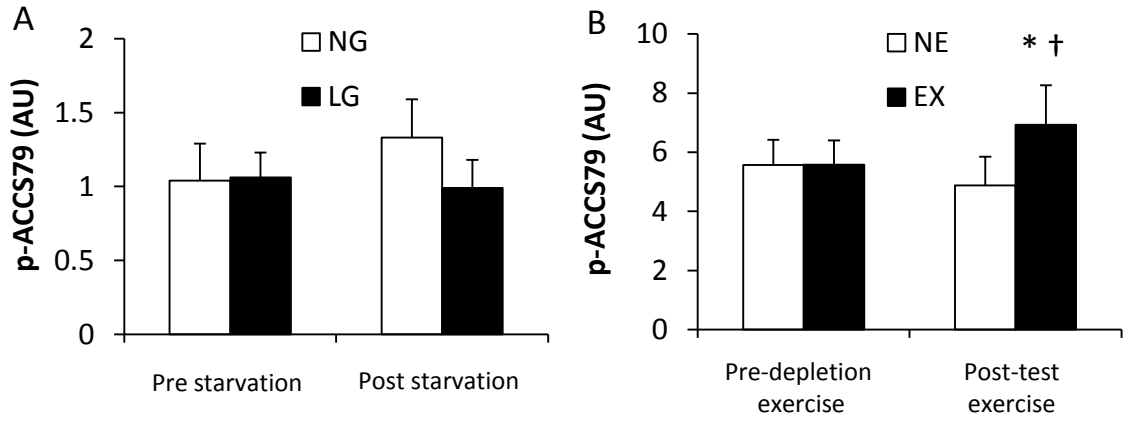

Figure 18. *Study I and III. The effect of the intervention on (A) p-ACC S^{79} protein levels in study III and (B) p-ACC S^{79} protein levels in Study I. Values are presented as means ± SEM. *p<0.05 vs pre; †p<0.05 vs pre.*

In Study III, ADP-stimulated mitochondrial respiration (state 3) with complex I substrate (malate + glutamate) decreased after starvation in both NE and EX (Figure 19a). When malate and glutamate were used with FA-derived complex I + II substrate (octanoyl-carnitine) and complex II substrate (succinate), respiration in EX was increased significantly

compared to pre starvation and NE. The elevated respiration in EX remained when the electron flux was uncoupled from ATP production (FCCP) and after inhibition of complex I with rotenone (Figure 19b). In study I, mitochondrial respiration, measured after sequential additions of ADP and substrates, was similar before and after exercise without difference between LG and NG (data not shown).

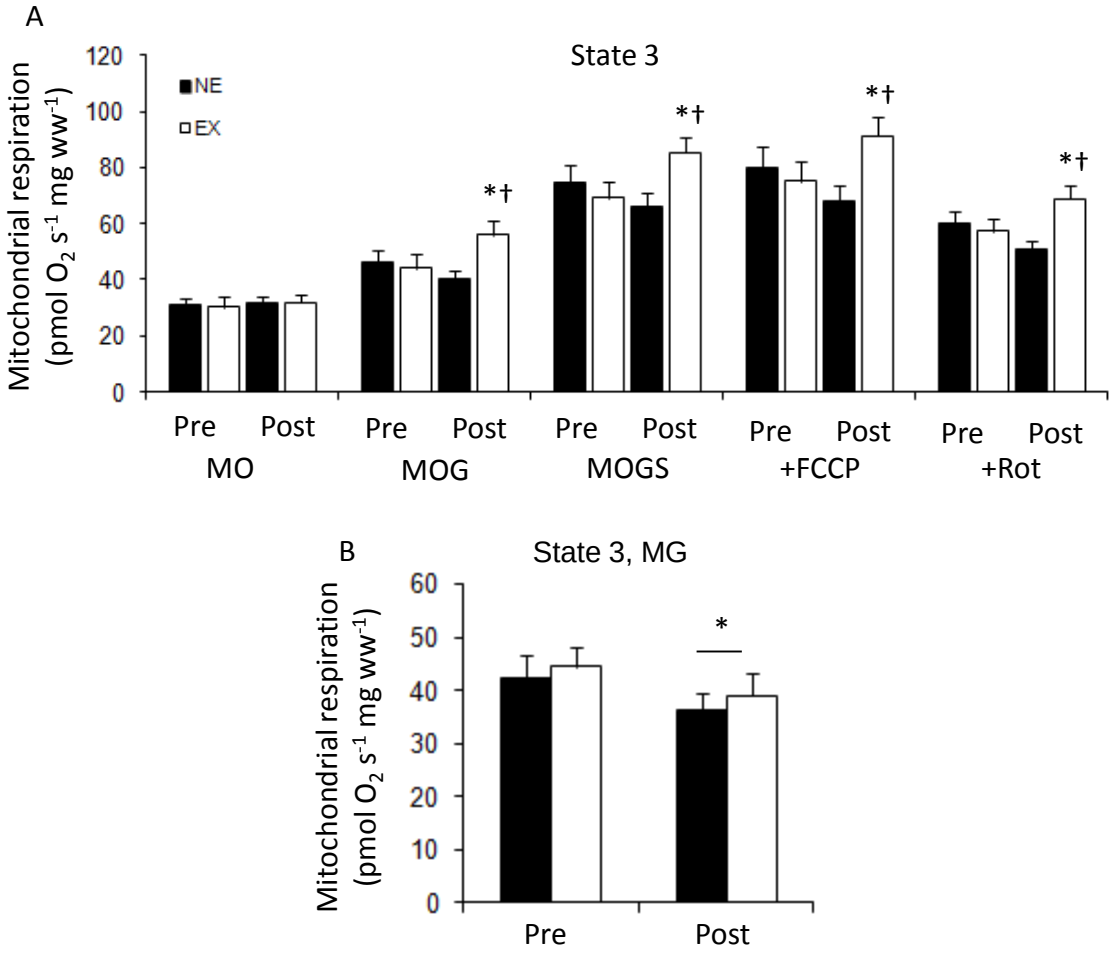

Figure 19. *Study III. Mitochondrial respiration. Muscle biopsies were obtained before (pre) and after (post) 75-h starvation with (EX) or without exercise (NE) (n = 9). Biopsies were taken prior to IVGTT. (A) ADP-stimulated respiration (state 3) with malate and octanoyl-carnitine (MO), MO+glutamate (MOG), MOG+succinate (MOGS), MOGS+FCCP (+FCCP), and MOGS+FCCP+rotenone (+Rot) (protocol 1). (B) ADP-stimulated respiration (state 3) with malate+glutamate (MG; protocol 2). Values are reported as means ± SE. *P<0.05 vs. pre; †P<0.05 vs. NE post; _*_ P<0.05, main effect of starvation vs pre.*

4.7 OXIDATIVE STRESS

In Study III, mitochondrial H_2O_2 production was reduced after starvation in both conditions with no difference between conditions (Figure 20a). In Study I, mitochondrial H_2O_2 production showed a strong trend (p=0.053) towards reduction 3 h after test exercise (Figure 20b). Glutathione status after test exercise also indicated reduced oxidative stress in that

GSSG/GSH ratio tended to be lower (p=0.076) (Fig. 20c). The exercise-induced trends of changes in mitochondrial H_2O_2 production and glutathione status were not different between LG and NG. Muscle content of MDA, a marker of lipid peroxidation, was not changed by exercise in neither condition (data not shown).

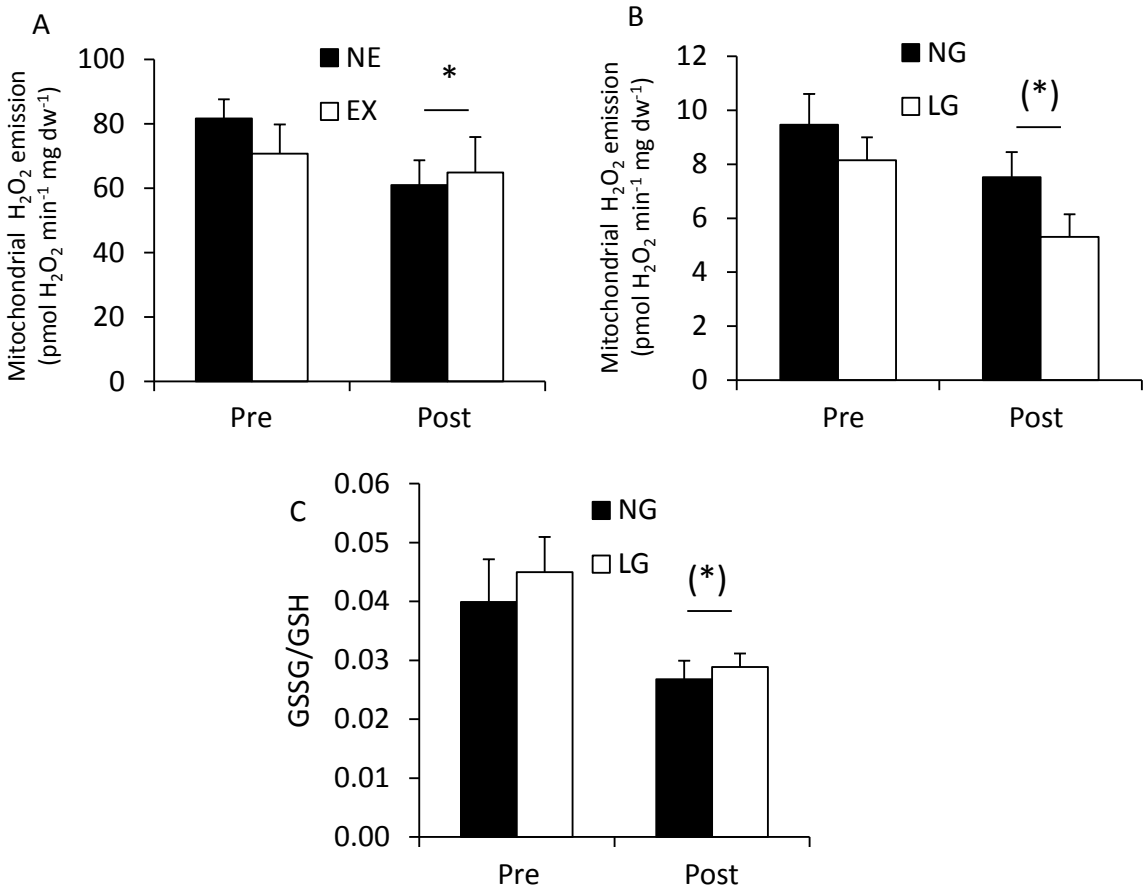

Figure 20. *Study I and III. Mitochondrial emission of H_2O_2 and GSSG/GSH. NE, no exercise; LG, low glycogen; NG, normal glycogen; EX, exercise; (A) mitochondrial H_2O_2 emission with octanoyl-carnitine+glutamate+succinate in study III (n = 9); (B) mitochondrial H_2O_2 emission with octanoyl-carnitine+pyruvate+succinate in study I (n = 8). (C) GSSG/GSH ratio in study I (n =5). Values are reported as means ± SE. * P<0.05, main effect of starvation vs pre; (*) P<0.05, trend for main effect of starvation.*

5 METHODOLOGICAL CONSIDERATIONS

5.1 IVGTT/OGTT

In Study III, glucose tolerance was measured with an IVGTT. Insulin sensitivity was then calculated using a method by Galvin, which correlates well with that obtained during euglycaemic hyperinsulinaemic clamp (54, 141). In Study IV, OGTT was used and insulin sensitivity was calculated using the Matsuda method, which also has been validated with the clamp method (2). However, there are certain limitations with both methods, such as unstable insulin levels, the inability to distinguish between insulin-dependent and insulin-independent glucose removal and between hepatic and peripheral insulin sensitivity (99). However, in contrast to the clamp, both OGTT and IVGTT provide information about the release of insulin in response to hyperglycaemia.

The difference between OGTT and IVGTT is whether the glucose bolus is ingested or infused. This results in different advantages with both methods. Ingesting the glucose is simple and mimics better the dynamics of physiological conditions, i.e. eating a meal. However, infusing the glucose gives more reliable plasma levels by bypassing gastro intestinal metabolism and absorption.

5.2 MITOCHONDRIAL RESPIRATION

The temperature during measurement in Study I was 25 °C and in Study III 37 °C. The reason for the lower temperature in Study I was concern about the stability of the mitochondria. However, following tests, long-term (five-hour) measurements at 37 °C only reduced respiration by ~20% and therefore the more physiological temperature was selected.

5.3 MARKERS FOR MITOCHONDRIAL CONTENT

Different markers for mitochondrial content were used in Study II and Study IV. In Study II CS activity was used and in Study IV the protein content of CS and OXPHOS complexes II, III and IV. Larsen and colleagues (89) correlated several markers for mitochondrial content with the golden standard, the transmission electron microscope. Of the methods we used, they found the strongest correlation for CS activity ($r=0.84$, $p<0.001$), then Complex II protein content ($r=0.73$, $p=0.005$), Complex III protein content ($r=0.61$, 0.03) and Complex IV protein content ($r=0.55$, $p=0.05$). They did not measure CS protein content. It is clear that in both Study II and Study IV, reliable markers for mitochondrial content were used.

5.4 WESTERN BLOT

Western blot is a method used all through the studies and it is therefore of importance. It is a well-known method that has been established for a long time at our lab. However, in the last study the method was modified. In Studies I–III the muscle homogenate was centrifuged to discharge myofibrillar debris. In Study IV, this step was disregarded to eliminate the possible loss of relevant proteins

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6 DISCUSSION

6.1 TRAIN LOW

Study I investigates the effect of exercise with low or normal levels of muscle glycogen on mitochondrial biogenesis. The subjects performed one depletion exercise, followed 14 hours later by a test exercise. In one condition they received carbohydrates following the depletion exercise session and in the other they did not. In the condition when they did not consume carbohydrates, glycogen levels were significantly lower compared to the other condition. The major finding in Study I was a profound increase in mRNA expression of PGC-1α following exercise with low glycogen levels. This may, over a training period, result in increased mitochondrial density and thereby improved performance.

In a more recent study Bartlett and colleagues confirm several of our findings (14). In their study, the timing of the biopsies was similar to ours, but the set up differed somewhat. A depletion exercise was only performed in the low glycogen group, and between exercise sessions the group fasted. They found that PGC-1α mRNA was up-regulated three hours post-exercise, which is the same as our study. In contrast to our study, PGC-1α was up-regulated prior to test exercise, 10–12 hours post-depletion exercise. In our study, pre-test biopsies were taken at least two hours later, and it is possible PGC-1α returned to basal levels during this time. However, a longer expression of PGC-1α in Bartlett and colleagues is reasonable considering the lower training level of the subjects and the different diet regime.

When glycogen levels are manipulated by an exercise and nutritional intervention, other metabolic changes will occur as a consequence. One difference between the groups was the calorie intake which was about three times larger in NG compared to LG. Calorie restriction has been shown to up-regulate PGC-1α and increase mitochondrial biogenesis (36, 50). However, these studies are performed over a longer period of time (six months), and over a period of 24-hour fasting it does not affect PGC-1α mRNA or protein levels (140). Insulin is a potent signal inducer and PGC-1α seems to play a role in insulin sensitivity (90). However, previous research shows that insulin does not suppress expression of PGC-1α two to five hours post-exercise (38, 110). Another possibility is the influence of elevated plasma FA. Prolonged (48-hour) lipid infusion suppresses PGC-1α mRNA (114). In contrast, fasting for 24 or 48 hours with increased circulating FA did not alter PGC-1α mRNA and protein expression (140). Altogether, it seems that the glycogen content during exercise, rather than calorie intake, plasma insulin or FA levels, is responsible for the pronounced increase in PGC-1α.

6.2 CONCURRENT EXERCISE

Study II investigates the effect of adding resistance training to endurance training on markers of mitochondrial content. The study was performed in trained subjects with cycling experience, and the aim was to maintain their regular exercise routines during the intervention in both groups, and then add resistance training to one of them. The study was

designed so two exercise sessions per week were exchanged for supervised training sessions at the lab; for the remaining week the subjects trained as usual. At these sessions both groups performed an endurance exercise session and then the ES group performed a session of strength training using the leg press machine.

Although the ES group increased strength (one RM during leg press), peak power output during 30 seconds of Wingate cycling and short-term endurance (TTE-VO_2max), they did not increase mitochondrial density (CS activity) or long-term endurance performance (TT40 and LT4). In contrast, the E group improved mean power during TT40 (+4%) and LT4 (+3%).

In study II we used a similar load (6 x 65–75%, one RM) during the resistance training, as in our previous study (145) and the one by Sale and colleagues (123). Training order, and strength prior to endurance, were also the same. Even so, our results correspond better with the study by Nelson et al (100) in which the subjects trained with a heavier load (3 x 6 RM) and performed the resistance exercise prior to the endurance exercise. In the study by Nelson et al the endurance group improved and the concurrent group did not. In addition, there was a significant difference between groups in CS activity and VO_2max. The explanation for the great responses following training is probably the low training levels of their subjects. However, the study included very few subjects (four in the endurance group and five in the concurrent group) and should therefore be interpreted with caution (100).

The lack of improvements in endurance performance and markers of mitochondrial biogenesis in Study II is probably, at least partly, due to the training level of the subjects. This is the first study to investigate the effect of concurrent exercise on markers for mitochondrial biogenesis in trained subjects. When investigated separately, resistance training seems to increase mitochondrial biogenesis in the untrained (124, 151), while in the trained, this is usually not the case (59, 151). This notion is also supported by Study IV, in which resistance training in the untrained elderly resulted in a profound increase in mitochondrial proteins. It is therefore reasonable that the outcome also depends on the training level of the subjects, when resistance training is combined with endurance training.

In contrast to our theory, long-term endurance performance did not increase after concurrent exercise training. Previous research in this area is contradictory, with studies finding an improved performance using heavy strength training with high loads (85–95%, 1 RM) and long durations (>8 weeks) (1, 119). It is possible that the strength training in our study was not heavy enough (65–75%, 1RM) or did not proceed long enough (eight weeks) to improve performance. In addition, most previous research used low or moderately trained subjects (19, 22, 64), while we used trained subjects. Similar to mitochondrial biogenesis, the effect on performance is most likely less profound in the trained. In another study that used trained subjects, concurrent exercise did not improve long term endurance performance (16).

In conclusion, we could not find any additional benefits of adding resistance to endurance training on mitochondrial density or endurance performance. For any potential benefit in

trained individuals, a longer period of training or alternative resistance training strategy is probably needed.

6.3 STRENGTH TRAINING IN THE ELDERLY

In Study IV elderly subjects (>65 years) performed heavy strength training (80%, 1RM) for eight weeks. The study is unique since it demonstrates a broad span of simultaneously occurring positive effects from resistance exercise training. The main findings of the study were that short-term resistance training in the elderly, in addition to improving strength, also improved glucose tolerance and muscle oxidative capacity.

Resistance training resulted in hypertrophy measured as increases in FFM, thigh circumference and thigh cross-sectional area. However, fiber CSA did not increase. Knee extensor strength for static, eccentric and concentric contraction increased by 8–12%. The increased RTD during the initial phase of contraction (0-30 ms), but not between 0-200 ms, is in line with previous findings (23). Increased muscle mass, strength and power are all well-known effects of resistance exercise in the elderly (72) and have important implications for health by prevention of fall injuries and increasing quality of daily life. The underlying molecular signaling pathways for muscle atrophy in the elderly are not fully understood. An interesting novel finding in the present study is the increased levels of signaling proteins involved in muscle protein synthesis (mTOR and Akt) in RET. The elderly have a reduced level of mTOR protein in muscle (42), which may restrict protein synthesis. Therefore, the observed increase in mTOR in RET may counteract any possible anabolic resistance and contribute to hypertrophy.

The most recognized test for the assessment of VO_2max and aerobic capacity is incremental exercise to fatigue, but the frailty of the subjects in this study prevented this test. Instead, changes in HR and RER during submaximal cycling were used as an indication of changes in aerobic capacity. The tendencies of decreased HR in RET and increased HR in CON suggest that resistance training improved VO_2max. Furthermore, the reduced RER at high intensity in RET, which indicate reduced lactic acid formation, support the view of improved aerobic capacity. The majority of previous studies (26, 52, 152), but not all (30), have shown that VO_2max increases after RET in aged subjects.

In addition to the improved whole body aerobic capacity, markers for muscular oxidative capacity improved. Fiber type composition shifted from fast twitch glycolytic Type IIx fibers towards more oxidative Type IIa fibers, and several mitochondrial proteins (OXPHOS complex II, IV and CS) increased markedly. Mitochondrial density and maximal respiration are reduced in the elderly, probably to a large part caused by physical inactivity (109). Endurance exercise training in the elderly is known to increase markers of muscle oxidative capacity i.e. OXPHOS protein content (56) and CS activity (87). However, the effect of RET is more contradictory. Studies show increased (52) or unchanged (47) CS activity. Mitochondrial volume measured with electron microscopy was increased in one study (80) and unchanged in another (139). Parise et al found that OXPHOS complex IV activity

increased with RET (105), and this is in similarity with the increased protein content in our study. Consistent with previous findings in young subjects (84), there were no changes in PGC-1α protein levels following RET. Although protein levels did not increase, we cannot conclude anything about the involvement of PGC-1α activity in mediating the mitochondrial biogenesis. The discrepancies between studies may depend on several factors like exercise set up, duration or analytical procedures.

The robust increases in mitochondrial proteins in Study IV after only eight weeks of training clearly demonstrates that resistance training in the elderly is an efficient strategy to improve muscle oxidative capacity. In addition, strength, power, HR during submaximal cycling and glucose tolerance improved. In contrast, the training status in the control group was impaired during the intervention period (reduced concentric torque, increased HR and RER during submaximal exercise). This is likely related to that the intervention period which occurred during autumn (September–November), which is a period when the degree of physical activity, especially in the elderly, is reduced.

6.4 INSULIN SENSITIVITY

In Study III, the effect of endurance exercise on starvation-induced insulin resistance was investigated. The main finding was that the impaired whole body insulin sensitivity and glucose tolerance after starvation were reversed or attenuated by exercise. Exercise was accompanied by reduced glycogen stores, increased lipid oxidation capacity, and alterations in signaling proteins involved in glucose and FA metabolism.

Consistent with previous studies, starvation resulted in hypoglycemia and reductions in plasma insulin, insulin sensitivity (34) and acute insulin response (49). In addition, plasma levels of FA and ketone bodies (i.e. BOH) increased markedly after starvation and demonstrate a metabolic state with increased lipid load. As mentioned previously, the surplus of lipids is associated with reduced insulin sensitivity and the mechanism has been attributed to an accumulation of toxic lipid intermediates and increased ROS formation (143). BOH, on the other hand, induces insulin resistance through an ROS-independent mechanism (156).

Glycogen levels are closely linked to insulin sensitivity (115). Even though muscle glycogen decreased significantly during starvation, insulin sensitivity was reduced. It appears that the level of glycogen depletion (reduced by 27%) was not sufficient to reverse insulin resistance. Previous research also shows slightly reduced glycogen levels appearing simultaneously with starvation-induced insulin resistance (34). Following exercise, the reduction in glycogen levels was more pronounced (reduced by 70%). Since post-exercise insulin sensitivity correlates with the amount of glycogen broken down during exercise (115), it is likely that the greater reduction is what caused the insulin resistance to reverse. Although the mechanism for how glycogen levels affect insulin sensitivity is unclear, it involves an increase in levels of membrane bound GLUT4 (115). GLUT4 is a glucose transporter and its presence in the membrane is a limiting factor for transmembrane glucose transport (Figure 21). The time period between exercise and biopsy was too short (three hours) for protein

translation to occur, and even so, levels of total GLUT4 were unchanged. Therefore the explanation is likely increased translocation of GLUT4 to the cell membrane. GLUT4 translocation to the surface membrane is stimulated synergistically by insulin and exercise, and the signaling cascades converge at Rab GTPase activating proteins, which include AS160 and TBC1D1 (33).

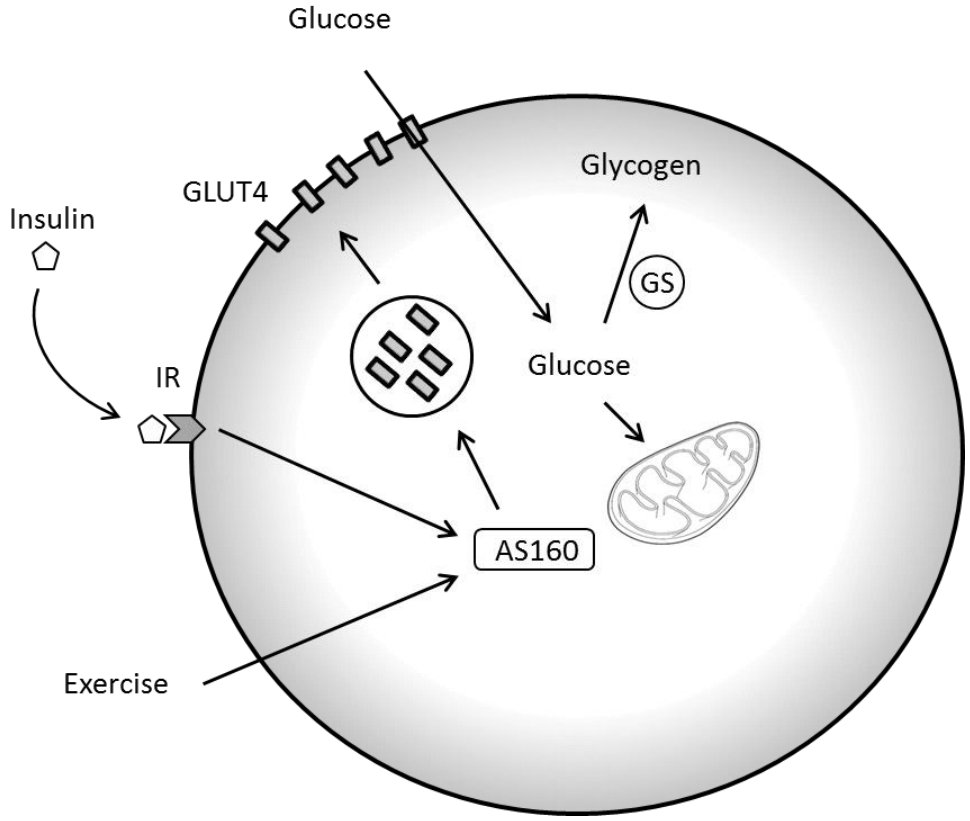

Figure 21. *Insulin and exercise stimulated GLUT4 membrane translocation and glucose uptake. IR, insulin receptor; GLUT4, glucose transporter 4; GS, glycogen synthase; AS160, Akt substrate of 160 kDa.*

Consistent with previous studies (131) starvation did not affect the basal levels of the insulin signaling molecules p-Akt and p-AS160 (using p-AS160-thr^{642}). However, previous research shows that starvation significantly decreases insulin-stimulated phosphorylation of Akt and AS160-thr^{642} (131). This suggests that starvation induces insulin resistance in human skeletal muscle by interfering with insulin signaling. In Study III AS160 phosphorylation was increased markedly (+138%) three hours after exercise, suggesting that the exercise-mediated enhancement of glucose tolerance and insulin sensitivity after starvation were related to enhanced glucose transport. It is likely that the increased AS160 phosphorylation was mediated by AMPK (even though p-AMPK was unaltered three hours post-exercise), as judged by elevated levels of the AMPK target p-ACC.

Impaired glycogen storage is considered to have a major role in insulin resistance (17, 129). Glycogen synthase (GS) is the rate-limiting enzyme for glycogen synthesis, and the activity is controlled by phosphorylation (inactivation) and dephosphorylation (activation), with insulin and low glycogen levels being factors that promote activation (117). Consistent with previous findings (34), Study III shows that starvation caused a significant increase in GS phosphorylation (i.e. inactivation). However, it was previously demonstrated that activation of GS during euglycemic hyperinsulinemia was not impaired after a three-day fast (34), indicating that starvation-induced insulin resistance is not caused by reduced GS activation. Prolonged exercise resulted in a marked dephosphorylation (i.e. activation) of GS and this has previously been shown by measurements of GS fractional activity as well (77). Thus it is possible that exercise-mediated activation of GS contributed to improved insulin action following starvation.

In Study IV, the long-term effect of resistance training on glucose tolerance was investigated in the elderly. Despite the short period of intervention (eight weeks) we observed an improved glucose tolerance in RET as shown by the reduction in $AUC_{glucose}$ and $Glu_{120\ min}$. This contention is supported by previous findings where glucose tolerance is improved by RET in the elderly (>65 years) with impaired glucose tolerance (122) or T2D (73), whereas the effect of long-term (four to six months) RET in healthy aged subjects show either an improvement (158) or no improvement of insulin sensitivity (44).

The increase in muscle mass after RET will increase the volume for glucose disposal and thus contribute to the increased glucose tolerance. In addition, resistance training increases insulin-mediated glucose uptake per unit of skeletal muscle, which indicates qualitative changes (5). An increased level of mitochondrial proteins is a qualitative improvement that may improve insulin sensitivity. As mentioned previously, increased mitochondrial density improves fat oxidation (69). This increases the ability of the mitochondria to cope with a high lipid load and may thereby improve insulin sensitivity.

In summary, starvation-induced insulin resistance may be caused by increased levels of lipids or BOH. During starvation, the mitochondrial capacity to produce ROS is reduced. When exercise is performed during starvation, insulin resistance is attenuated. The effect is associated with reduced glycogen levels and activation of GS and AS160, which are possible mediators of the effect. Although resistance training in the elderly improves glucose tolerance, the effect seems more pronounced in individuals with impaired glucose tolerance. The effect may, at least partly, be explained by increased amounts of mitochondrial proteins.

6.5 MITOCHONDRIAL RESPIRATION AND FA METABOLISM

In Study III, phosphorylation of ACC and mitochondrial respiration with Complex I+II substrates was increased following exercise during starvation. Lipid oxidation is in part controlled by mitochondrial uptake of long-chained FA via (carnitine palmitoyltransferase I) CPT-1. Malonyl-CoA inhibits CPT-1 and the formation of malonyl-CoA is controlled by the activity of ACC. Phosphorylation inhibits ACC, which decreases the formation of malonyl-

CoA and thereby stimulates mitochondrial FA uptake (153). However, the increase in p-ACC cannot explain the increased mitochondrial respiration with Complex I+II substrates *in vitro*. The substrate used, octanoyl-carnitine, is a medium-chain FA and therefore not transported by CPT-1 (28). Even so, increased phosphorylation of ACC and increased mitochondrial respiration with complex I+II substrates suggest that there is an increased capacity for the mitochondria to transport and oxidize lipids after exercise.

Study III confirms previous research that shows an increase in p-ACC following exercise (146, 154). However, within three hours the amount of p-ACC has returned to basal levels in both trained and untrained subjects (146, 154). It seems that the metabolic state in Study III, i.e. increased plasma FA and reduced glucose availability, elongated the ACC phosphorylation. In contrast, p-ACC did not change despite a similar metabolic state in Study I. This is most likely due to the training level of the subjects, with a more prolonged response in the untrained subjects in Study III. Phosphorylation of the upstream regulator of ACC, AMPK, was not affected in either study. As with p-ACC, p-AMPK levels return to basal within three hours, and it is likely we missed that window due to the timing of the biopsies (146, 154).

In Study III starvation resulted in reduced mitochondrial ADP stimulated respiration with Complex I substrates. In a similar study, 60 hours of fasting resulted in decreased respiration with Complex I+II substrates as well as during maximal uncoupled conditions (67). A novel interesting finding was the increase in mitochondrial Complex I+II substrate oxidation when starvation was followed by exercise. It has previously been shown that both high (three intervals at 130% VO_2max to exhaustion) and moderate (70% VO_2max) intensity can increase State 3 respiration in permeabilized fibers for at least three hours (130, 138). When exercise is performed during starvation there is an increased reliance on fat oxidation (82); this study shows that the effect is sustained on a mitochondrial level for at least three hours. Since respiration with Complex I substrates was unaffected by exercise in Study III, the results demonstrate a substrate-specific mitochondrial adaption. Although exercise can increase Complex I respiration (130, 138), Complex I+II respiration is more pronounced following exercise in states of high lipid availability, e.g. high-fat diet or ultra-endurance exercise (46, 130). Considering that both mitochondrial respiration and p-ACC increased in Study III, it shows that exercise-induced mitochondrial FA metabolism was creased in Study III.

In contrast to Study III, exercise did not increase mitochondrial respiration capacity in Study I, even though the subjects had low plasma glucose, glycogen and high plasma FA. Neither was p-ACC increased. They performed a similar exercise session at moderate intensity in repeated 10 minute intervals. The reason for this is likely the training level of the subjects. In previous research, as in Study III, the subjects were recreationally active (130, 138) while in Study I, the subjects were well trained.

6.6 ROS

The mitochondrial ROS production capacity was reduced in Study III, and showed a trend towards reduction in Study I. Although acute exercise may alter mitochondrial respiration (130, 138), ROS production capacity is unaffected three hours following moderate intensity exercise under the current conditions. In Study I, there was a trend towards reduced GSSG/GSH, and MDA, a marker for lipid peroxidation, was unaffected. The exercise was performed at an intensity of 72.5–60% of VO_2max, and previous research shows that oxidative stress predominantly occurs at higher intensities (48, 92). Even though several of the subjects in both studies felt exhausted and could not finish the exercise session at the initial intensity, it seems that the intensity was too low to induce oxidative stress.

Although the exercise intensity and duration can explain the lack of increases in ROS production capacity and GSSG/GSH, it does not explain why they decreased. Previous research shows that a state of energy or nutrient deficiency reduces ROS production in mice (127). In both Study I and Study III, plasma glucose and muscle glycogen were reduced and FFA increased. Plasma FFA increased in all groups except NG in Study I. Since ROS production also was reduced in that group, FFA is probably not the cause. In addition, high levels of FA are associated with increased ROS production (7).

Statistical analyzes were performed with two-way repeated measures ANNOVA, and no differences in ROS production was found between groups. However, when the ROS production in Study I was analyzed with a T-test for each group separately, it was reduced in LG ($p=0.019$) but not in NG. This can be interpreted as a more pronounced reduction in ROS production in LG. LG differs from NG and Study III by having exogenous glucose provided, which increased glycogen levels and nearly returned plasma glucose to basal levels. High glucose availability increases ROS production in muscle cells (97) and it is possible that ROS production is attenuated by low glucose availability. This, however, needs further research.

In summary, we could not find any signs of oxidative stress mediating the pronounced increase in PGC-1α mRNA in Study I. It is likely that during moderate-intensity exercise with low glycogen levels, PGC-1α mRNA is predominantly regulated by other signals. In addition, moderate-intensity exercise, even when performed to near exhaustion in a state of reduced glycogen stores, is not sufficient to increase mitochondrial ROS production capacity. However, starvation and long-term (>17 hours) reduction in glycogen stores is associated with reduced mitochondrial ROS production capacity.

7 CONCLUSION

- Training with low glycogen levels is an efficient strategy to increase mRNA levels of PGC-1α, a marker for mitochondrial biogenesis. The effect is likely caused by reduced glycogen levels rather than plasma insulin, plasma FA or oxidative stress. This shows that training with low glycogen stores may increase mitochondrial content and thereby performance.

- Adding resistance training to endurance training did not improve mitochondrial density or endurance performance in trained individuals.

- Resistance training over a short period of time is an efficient strategy to improve strength, aerobic capacity (HR during submaximal cycling) and glucose tolerance. The improvements may, at least partly, be explained by an increased amount of mitochondrial and hypertrophy signaling proteins.

- Starvation induces insulin resistance, and when exercise is performed, the insulin resistance is reversed. This is likely explained by reduced glycogen stores, increased p-AS160 and reduced p-GS.

- Starvation reduces mitochondrial respiration with Complex I substrates and ROS production capacity. When exercise is performed during starvation, p-ACC and mitochondrial respiration capacity with FA substrates are increased.

- Moderate-intensity exercise, even when performed to near exhaustion in a state of reduced glycogen levels, seems not to affect mitochondrial ROS production capacity or oxidative stress.

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